Studies on defective interfering Semliki Forest Virus

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Declaration

I hereby declare that this thesis has been composed by myself and has not been used in any previous application for a degree. All the work has been carried out by myself except for some of the infectivity titrations in Chapters 3 and 4 of the Results which were performed in collaboration with Alan Guest and Terri Atkinson, and the T_1 oligonucleotide fingerprint in Figure 21 was done in collaboration with Dr. Jon Clewley.

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Abbreviations

cpe	cytopathic effect
DI	defective interfering
h	hours
i.c.	intracerebral
IFN	interferon
i.n.	intranasal
i.p.	intraperitoneal
moi	multiplicity of infection
M_r	molecular weight
NCS	newborn calf serum
p-	passage
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming unit
p.i.	post-infection
RNA	ribonucleic acid
mRNA	messenger RNA
S	standard
sec	seconds
TCA	trichloroacetic acid
UV	ultra-violet
EMC	encephalomyocarditis
LCMV	lymphocytic choriomeningitis virus
SFV	Semliki Forest virus
SV	Sindbis virus
VSV	Vesicular stomatitis virus

General Introduction

1. SEMLIKI FOREST VIRUS (SFV)

a) History

Semliki Forest virus (SFV) was originally isolated from a pool of 130 female *Aedes abnormalis* mosquitoes captured in Bundinyama, Uganda in 1942 (Smithburn and Haddow, 1944).

b) Classification

SFV is classified as a member of the genus alphavirus, of the family Togaviridae (Matthews, 1982). It was originally described as an arbovirus ("arthropod-borne" virus) (Casals and Brown, 1954) since it infects vertebrates and is transmitted by mosquito vector. However, later studies showed that the biochemical properties of the arboviruses were variable. For example, some members of the famiy Bunyaviridae were originally classified as arboviruses and these have a genome of three (negative sense) RNA segments whereas SFV has positive sense genomic RNA.

A large number of the arboviruses were found to be morphologically similar, sharing certain structural and biochemical characteristics. These were grouped together and termed togaviruses (toga = shroud, cloak, envelope) (Andrewes, 1970). Two major serologically unrelated genera of the Togaviridae were recognised and were termed group A and group B. These were later designated the genera alphavirus (e.g. SFV) and flavivirus (e.g. yellow fever virus) (Wildy, 1971. Later, two

other genera of the family Togaviridae were also recognised and these are the rubiviruses (e.g. rubella virus) and pestiviruses (e.g. hog cholera virus) and, to date, there are also five unclassified viruses.

Using the Baltimore classification scheme (Baltimore, 1971) for viruses, togaviruses are members of group IV since they contain a single RNA genome of positive polarity (i.e. it is the same sense as mRNA).

SFV is relatively non-pathogenic for man and has been used as a model system for studying alphaviruses. Other alphaviruses are pathogenic for man and cause serious, sometimes fatal, diseases (e.g. Eastern (EEE), Western (WEE) and Venezuelian (VEE) equine encephalitis viruses) and it is only recently that studies on these viruses have been reported.

c) Hosts and Geographical Distribution

SFV is found throughout much of Africa and has been isolated from mice, birds and several species of mosquitoes (Berge, 1975). However, serological studies suggest that the virus is far more widespread than just Africa. Neutralising antibodies have been found in man in Africa, India, Malaya, Borneo, Vietnam, Thailand and the Philippines (Berge, 1975). To date only one human, a laboratory technician in Germany, is known to have developed clinical illness due to SFV and this resulted in a fatal encephalitis (Willems et al., 1979). There is no information about the natural vector-host cycle of the virus.

d) Pathogenesis

SFV was originally discovered by the ability of mosquito extracts to cause disease in mice (Smithburn and Haddow, 1944), and it is on this animal host that the majority of studies on the pathogenesis of the virus have been performed. The original study showed that intracerebral inoculation of SFV caused paralysis in one mouse (out of 6 inoculated) after 27 days incubation. However, a brain suspension taken from this mouse caused sickness after only 4 days incubation in other mice after intracerebral inoculation. These original studies also showed that the virus was lethal for guinea-pigs, rabbits and rhesus and red-tailed monkeys after intracerebral inoculation. This was confirmed by Henderson et al. (1967), Boulter et al. (1971) and Bradish et al. (1971) who also showed that the virus caused infection after inoculation by various routes. Examination of tissues from infected animals showed that the virus and pathology were mainly in the brain, hence the virus is described as being neurotropic.

Smithburn and Haddow (1944) also showed that the virus became adapted to mice after passage by intracerebral inoculation and the incubation period and average survival time of infected mice decreased during passage. The latter was only 2.6 days at passage 21. Thus, most laboratory strains of SFV are mouse pathogenic derivatives of the original isolate. Bradish and co-workers have made use of this fact to derive variants of SFV with a gradient of low, intermediate and high neurovirulence (Bradish et al., 1971). These variants were found to have equal particle to infectivity ratios in tissue culture and were lethal to suckling mice. Differences in the variants were seen in their

virulence for mice of 15 days of age or older. "Virulent" variants killed mice, while "avirulent" variants did not. Many complex experiments have shown that the virus populations are heterogeneous in their neurovirulent properties for adult mice (Bradish et al., 1971, 1972; Bradish and Allner, 1972). However, these studies have failed to show which properties of the virus determine virulence.

Studies on the distribution of virus, in mice infected by different virus variants, showed that virus titres were similar for the first hours after infection and it was only after approximately 48 h that differences became apparent. Virulent variants were found to show higher infectivities than avirulent variants. Also the rate of growth of virulent virus was greater than avirulent virus (Pusztai et al., 1971; Bradish and Allner, 1972; Fleming, 1977). Woodward and Smith (1979) have reported that avirulent virus is temperature sensitive, but the significance of this finding is unknown. Recently, Atkins (1983) has shown that the avirulent and virulent virus did not differ in adsorption to cells, nucleocapsid synthesis, protein synthesis, the ratio of 42S to 26S RNA, particle to infectivity ratio, interferon induction and defective interfering particle production; however, avirulent virus did synthesise less total viral RNA but was incorporated into virions more efficiently than virulent virus. Thus, it is possible that high virulence is associated with a high rate of virus replication, such that virus growth exceeds the build up of immune responses. It is also possible that the slower replication of avirulent virus allows the intervention of the host immune response to limit the avirulent virus infection or that avirulent and virulent virus infect different cells of

the central nervous system (see below).

In general, virulent virus causes a fatal encephalitis in young mammals, while avirulent virus produces a focal self-limiting encephalitis which is usually sub-clinical. However, Atkins et al. (1982) have reported that the avirulent virus will kill embryos in pregnant mice. Also using the intraperitoneal route of inoculation, it has proved possible to convert avirulent virus infection into virulent virus infection by blockading the phagocytic function of macrophages with colloidal gold (Allner et al., 1974; Oaten et al., 1982). The reason for this change in pathogenesis is unknown at present.

Both virulent and avirulent virus cause distinct histopathological lesions in the brains of infected mice. Virulent strains affect neurones and oligodendrocytes causing acute degenerative changes, including neuronal necrosis, while avirulent strains cause demyelination by destruction of oligodendrocytes (Mackenzie et al., 1978; Suckling et al., 1978; Barrett et al., 1980; Sheahan et al., 1981; Atkins and Sheahan, 1982).

The pathogenesis of demyelination in SFV infection is controversial. The work of Atkins and colleagues suggests that it is the direct cytopathic effect of virus on oligodendrocytes that causes demyelination and the host immune response is of secondary importance, while the group of Webb et al. propose that the host immune response is responsible for the lesions in the brain. The situation has yet to be clarified.

Atkins' experiments centre on using mutagenesis to isolate neurovirulent mutants of the virulent L10 strain (Barrett et al., 1980) and to study the effect of the mutation on the development of the disease in the host. Atkins and Sheahan (1982) have presented evidence that demyelination is associated with the destruction of oligodendrocytes. Later studies (Sheahan et al., 1983) also suggest that virus induced injury to oligodendrocytes plays a major role in the cause of demyelination and that the immune response is also important, but plays a subsiduary role.

On the other hand, Webbs' group has concentrated on studying the pathogenesis of the avirulent A774 strain. They propose that demyelination is immune mediated (Jagelman et al., 1978; Suckling et al., 1978; Berger, 1980; Kelly et al., 1982). Jagelman et al. (1978) reported that the brains of athymic "nude" (nu/nu) mice (impaired T cell responses and production of thymus dependent antibody) have no lesions yet heterozygous litter mates (nu/+) do have lesions. However, Chew-Lim (1979) finds that inflammatory reactions in nude mice were not absent, but merely less severe than immunocompetent mice. Bradish et al. (1979) reported that nude mice had an encephalitis similar to nu/+ mice, while Berger (1980) showed that nude mice had a reduced brain pathology compared with mouse controls. Recently, Fazakerly et al. (1983) have transferred normal immune spleen T cells to nude mice and reconstituted the brain pathology. Clearly the results are conflicting. Supportive evidence for immune mediated lesions is the failure to detect virus particles in oligodendrocytes (Chew-Lim, 1975; Chew-Lim et al., 1977; Pathak et al., 1976; Pathak and Webb, 1978; Kelly et al., 1982).

Although the two groups have conflicting results, these may in part be explained by the use of neurovirulent mutants by Atkins and avirulent A774 strain by Webb which may give different responses in mice.

Finally, Zlotnik et al., (1972) have reported on delayed pathological changes in mice which had survived infection with the avirulent strain A8. Mice initially had brain lesions but these were undetectable at 6 weeks pi, however at 2 years pi they developed extensive pathology in brain. Unfortunately, this study has not been confirmed and the significance has yet to be assessed.

e) Structure

SFV is an enveloped virus with an overall diameter of approximately 55nm. The virus consists of an icosahedral nucleocapsid surrounded by a lipid bilayer (Murphy, 1980) and has four structural proteins. The nucleocapsid consists of the genome surrounded by capsid (C) protein. Electron microscopy, sucrose gradient centrifugation and PAGE have shown that the genome consists of one single-stranded RNA molecule of sedimentation coefficient 42S (Friedman et al., 1966) and a M_r of 4.3 x 10^6 (Simmons and Strauss, 1972b; Simmons and Strauss, 1972a and Hsu et al., 1973). The genome is infectious (Sonnabend et al., 1967) and can thus act as a mRNA. At the 3' end of the genome there is a poly A tract of approximately 60 nucleotides (Armstrong et al., 1972; Eaton and Faulkner, 1972) and a cap structure (an inverted 7-methyl guanosine) has been found at the 5' terminus (Hefti et al., 1976; Dubin et al., 1977; Pettersson et al., 1980). The C protein has a M_r of 30,000 and has a

clustering of basic amino acids and prolines near the amino terminus. These are thought to be involved in binding of RNA in the nucleocapsids (Garoff et al., 1980a).

The lipid bilayer has external glycoprotein spikes each consisting of three different glycosylated polypeptides known as E1, E2 and E3, of M_r's 49,000, 52,000 and 10,000 respectively (Simons et al., 1973; Garoff et al., 1974; Ziemiecki and Garoff, 1978). E1 contains the determinants for haemagglutination and haemolysin activity, while E2 has the determinant for neutralization (Dalrymple et al., 1976; Chanas et al., 1982). E3 has only been found in SFV and it is thought that E3 is released into the extracellular fluid by other alphaviruses (Welch and Sefton, 1979). The aminoterminal region of E3 functions as a signal sequence for the E3-E2 precursor protein p62 (see below, Section I.f.vi, "Translation of structural proteins") (Garoff et al., 1980a).

It is thought that E1, E2 and E3 exist as a trimer and form the spike-like structures observed on the surface of the virus (Ziemiecki and Garoff, 1978). E2 spans the lipid bilayer with approximately 30 amino acids present on the internal side of the membrane (Garoff and Soderlund, 1978; Garoff et al., 1980a) such that the hydrophobic carboxyterminus interacts with the nucleocapsid. E1 is attached to the lipid bilayer by its hydrophobic carboxyterminus and E3 is thought to be inserted on the extenal side of the membrane (Uterman and Simons, 1974; Garoff and Simons, 1974; Pfefferkorn and Shapiro, 1974; Strauss and Strauss, 1977; Ziemiecki and Garoff, 1978; Garoff and Soderlund, 1978). In BHK cells, E1 and E3 contain one oligosaccharide unit each,

while E2 has two (Mattila et al., 1976; Pesonen and Reskonen, 1976, 1977). The lipid bilayer is of host cell origin (Pfefferkorn and Hunter, 1963). All the structural proteins are present in equimolar quantities of 240 molecules per virion (Laine et al., 1973; Garoff et al., 1974) and the overall chemical composition of the virus is 6.3% RNA, 12.2% C protein, 44.4% envelope proteins, 30.8% lipid and 6.3% carbohydrate (Laine et al., 1973). To date, the Togoviridae is the only family of enveloped virus to have only one protein in the nucleocapsid. The majority of enveloped viruses have an additional internal protein usually known as the matrix or M protein.

f) Multiplication

A scheme for the replication cycle of SFV is shown in Figure 1.

i) Multiplication

SFV multiplies entirely in the cytoplasm of the cells it infects (Grimley et al., 1968). It grows in a wide variety of cell types and over a wide temperature range of between 20 to 41°C (Pfefferkorn and Shapiro, 1974). At 37°C the growth cycle normally takes 6 to 10 h (Dulbecco and Vogt, 1954); whereas at lower temperatures of 27 to 29°C this time can be doubled (Burge and Pfefferkorn, 1966). In the majority of cases, the replication of the virus causes a severe cpe usually resulting in death of the host cell within 10 to 20 h at 37°C (Hardy and Brown, 1961; Acheson and Tamm, 1967; Erlandson et al., 1967). The host cell DNA, RNA and protein synthesis are rapidly and extensively inhibited within 3 to 5 h pi by mechanisms at present not understood

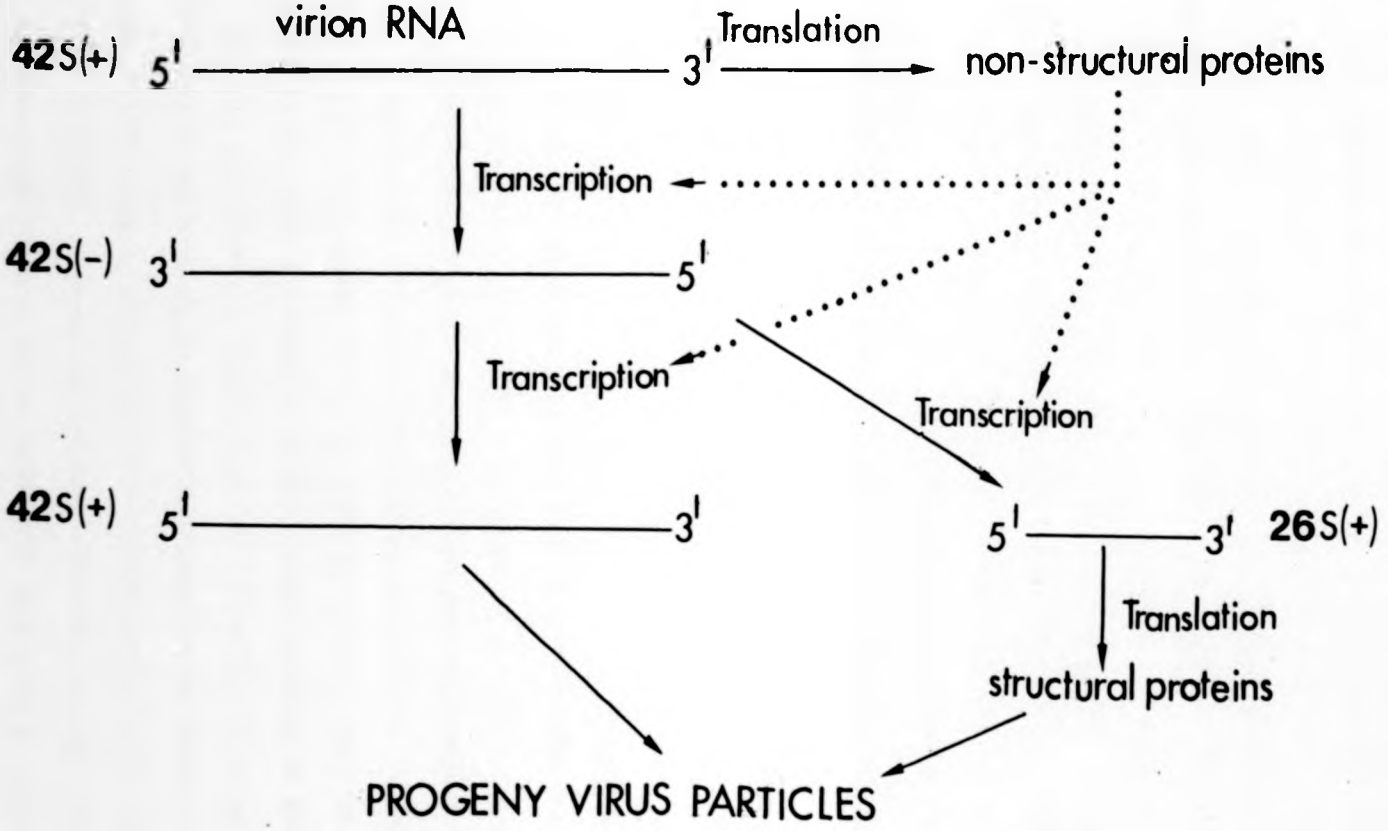

virion RNA
42S(+) 5' 3'
Translation
non-structural proteins
Transcription
42S(-) 3' 5'
Transcription
Transcription
42S(+) 5' 3'
5' 3' 26S(+)
Translation
structural proteins
PROGENY VIRUS PARTICLES

Figure 1. Diagrammatic representation of the replication cycle of SFV

(Taylor, 1965; Lust, 1966; Strauss et al., 1969; Mussgay et al., 1970; Mantani and Kato, 1975; Atkins, 1976; Simizu et al., 1976; Wengler, 1980). During a lytic infection as many as 20,000 progeny virus particles are released per cell (Pfefferkorn and Shapiro, 1974). Multiplication of the virus in invertebrate cells at 28°C has a slower cycle of 12 to 24 h (Igarashi et al., 1977; Stollar et al., 1975) and there is often little or no cpe or inhibition of host macromolecular synthesis (Peleg, 1969, 1972; Stevens, 1970; Logan, 1980; Stollar, 1980). These infections often become persistent, with only a few cells producing virus (Stollar et al., 1973; Shenk et al., 1974; Stollar, 1980). However, Stollar et al. (1973) have reported a cytolytic infection of cloned mosquito cells. Recent studies have suggested that host transcription is required for the replication of alphaviruses in both vertebrate and arthropod cells, but this finding has yet to be elucidated (Sheefers-Borshel et al., 1981; Baric et al., 1983; Erwin and Brown, 1983).

ii) Uptake of the virus

The nature of the receptor for adsorption of the virus into the cell is still unknown. However, removal of the virus spikes leads to inactivation of the virus, suggesting that the envelope proteins play an essential role in attachment of the virus to cells (Osterrieth, 1965; Compans, 1971; Sefton and Gaffney, 1974; Uterman and Simons, 1974). Current theories propose that SFV entered the cell by receptor mediated endocytosis (Fan and Sefton, 1978; Helenius et al., 1980) and uncoating is thought to occur in endosomes by the fusion of the virus and endosome

membranes when the pH falls below 6 (Helenius et al., 1980; Marsh and Helenius, 1980; White and Helenius, 1980; White et al., 1980; Marsh et al., 1983). It has been suggested that SFV has multiple receptors for binding and in BHK-21 cells the virus preferentially binds to microvilli (Fries and Helenius, 1979; Helenius et al., 1980). Since SFV has a wide host range it must either have a wide range of cell surface receptors or one which is evolutionarily conserved. Helenius et al. (1978) support the latter view suggesting that the virus binds to proteins encoded by the major histocompatibility complex, but Oldstone et al. (1980) have reported that these are not the only proteins which function as receptors for SFV.

iii) Translation of non-structural proteins

Once inside the cell, the 42S RNA^+ genome is translated to produce the non-structural proteins which form the virus polymerase. Translation of 42S RNA begins at an initiation site two-thirds of the length down the molecule. The precursor polypeptide produced is then cleaved to yield the various non-structural polypeptides, i.e. the non-structural polypeptides are produced by post-translational cleavage (Lachmi and Kääriäinen, 1976; Brzeski and Kennedy, 1977; Glansville et al., 1978; Fuller and Marcus, 1980a; Lehtovaara et al., 1980; Collins et al., 1982).

The components of the virus RNA polymerase have not yet been well characterised. Studies with temperature sensitive mutants of SV suggest that the polymerase consists of four viral polypeptides (Strauss et al.,

1976) (but see Clewley and Kennedy, 1976, below), and in fact four non-structural polypeptides have been detected for SFV of M_r's 70,000 (ns70), 86,000 (ns86), 72,000 (ns72) and 60,000 (ns60) (Lachmi and Kääriäinen, 1976, 1977; Glanville and Lachmi, 1977; Glanville et al., 1978; Lehtovaara et al., 1980; Keränen and Ruohenen, 1983). From these studies the order of translation was proposed as being NH_2-ns70-ns86-ns72-ns60-COOH, however recent studies suggest it is NH_2-ns70-ns86-ns60-ns72-COOH (Keränen and Ruohonen, 1983) Clewley and Kennedy (1976) have reported the isolation and purification of the SFV polymerase and showed that it contains only two virus-specific non-structural polypeptides of M_r 63,000 and 90,000 and possibly also host proteins. Clearly the results differ, but may in part reflect technical differences in PAGE. Kennedy et al. used the system of Laemmli (1970), while Kääriäinen et al. used that of Neville (1971) which resolves more non-structural polypeptides. Indeed, Keränen and Ruohonen (1983) failed to detect all the non-structural proteins of SFV using the Laemmli system.

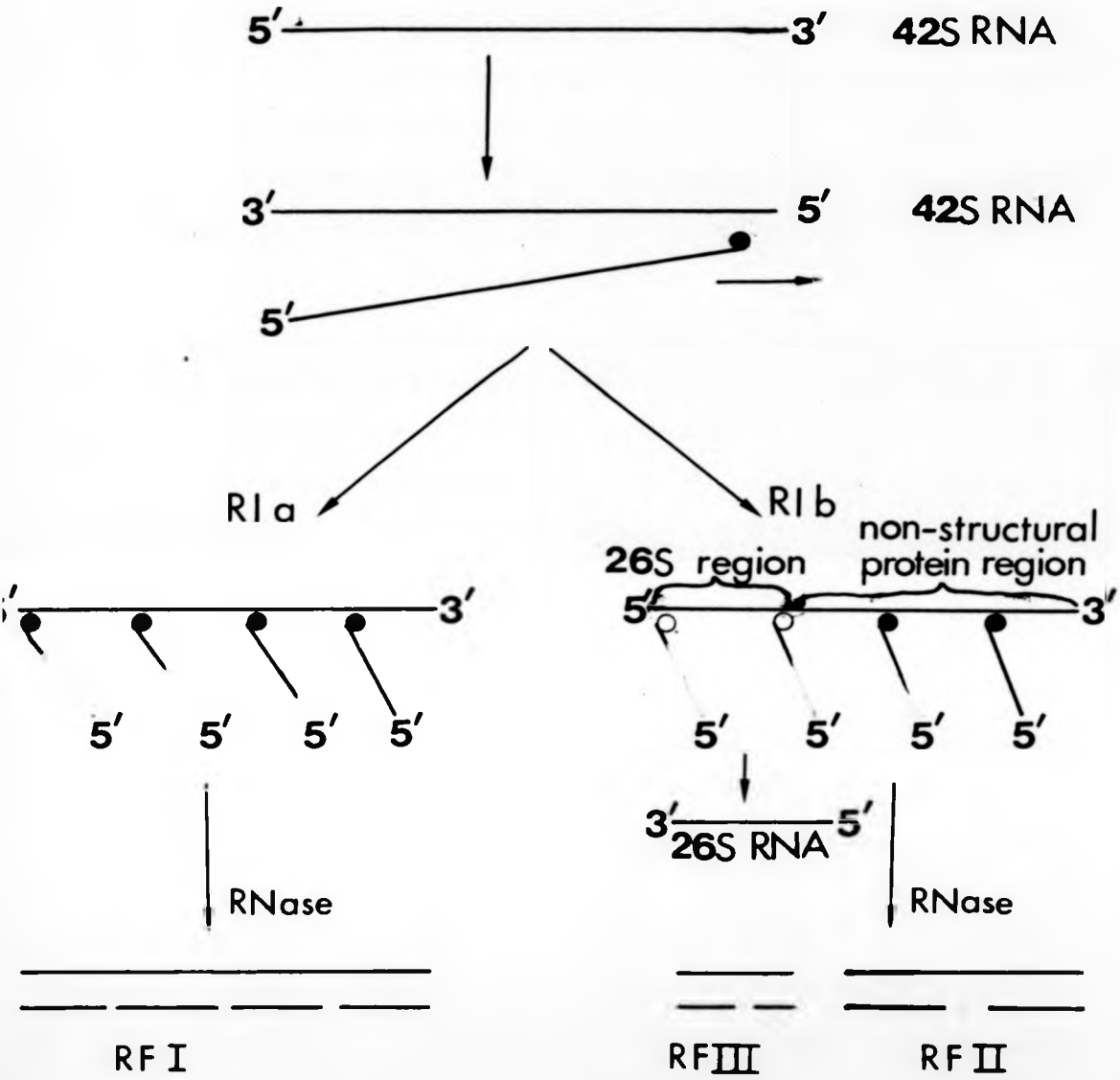
iv) RNA synthesis

The first RNA species to be synthesised in infected cells is the complementary strand of the genome (i.e. 42S RNA^-). This is used as the template for the synthesis of two species of RNA^+, either new genomes (42S RNA^+) or a subgenomic 26S RNA^+, which codes for the four structural polypeptides of the virus. Synthesis of 42S RNA^- begins at about 1 h pi, and reaches a maximum rate at about 2.5 h, and ceases by 4 h pi (Bruton and Kennedy, 1975; Sawicki and Sawicki, 1980). In contrast,

the rates of syntheses of both 42S RNA^+ and 26S RNA^+ are constant throughout the replication cycle (Bruton and Kennedy, 1975; Sawicki and Sawicki, 1980). It is thought that the regulation of RNA synthesis is controlled by the virus polymerase since the virus polymerase synthesising RNA^- has a shorter half life than that controlling RNA^+ (Sawicki and Sawicki, 1980; Sawicki et al., 1981).

The major mRNA species in infected cells is 26S RNA. Hybridization studies have shown that this RNA (M_r 1.6 x 10^6) represents one-third of the 42S RNA (Simmons and Strauss, 1972a), oligonucleotide mapping has shown that the 26S RNA represents the 3' end of the 42S RNA (Kennedy, 1976; Wengler and Wengler, 1976) and sequence studies have shown the 26S RNA and 42S RNA are co-terminal (Ou et al., 1981). The 26S RNA has a poly A tract at the 3' terminus and is capped at the 5' terminus (Dubin et al., 1979; Pettersson et al., 1980). Recent studies have shown that the transcription of 26S RNA takes place by internal initiation rather than by a "jumping" polymerase (Pettersson et al., 1980).

The 42S RNA is synthesised in a double-stranded replicative intermediate structure (RI_A), which after mild ribonuclease treatment is converted to a pseudo double-stranded form of 42S RNA consisting of a core of complementary strands (RNA^+ and RNA^-), known as replicative form, RFI (M_r 8.8 x 10^6). The 26S RNA is synthesised in a different replicative intermediate (RI_b) which is converted to RFII (M_r 5.6 x 10^6), a duplex of the 5' two-thirds of the genome, and RFIII (M_r 3.2 x 10^6), a duplex of the 26S RNA (Simmons and Strauss, 1972a,b; Kennedy, 1976; Wengler



● RNA polymerase

○ RNA polymerase (internal initiation)

Figure 2. Model for the transcription of SFV RNA

RNase is ribonuclease.

and Wengler, 1976) (see Fig. 2). Recently sequence studies have confirmed the RFI structure and also shown that the 5' cap is absent from 42S RNA^+ (Wengler et al., 1982b).

Other single-stranded RNA species have been detected with sedimentation values of 38S and 33S (Simmons and Strauss, 1974; Kääriäinen and Gomatos, 1969) but these have been shown to be conformational variants of the 42S and 26S RNA species respectively (Simmons and Strauss, 1972a, 1974; Kennedy, 1976; Wengler and Wengler, 1976).

v) Genome sequence and structure

Recently, the majority of the SFV genome has been sequenced (the genome consists of approximately 12,700 nucleotides, Simons et al., 1982) and comparison with other alphaviruses has identified regulatory elements important in the replication of alphaviruses. Four distinct control elements have been recognised (see Fig. 3). On the 42S RNA^+ strand, at the 3' terminus, adjacent to the poly A tract, a 19 nucleotide sequence is thought to be the polymerase recognition site for initiation of RNA^- synthesis (Ou et al., 1982b). Part of a 51 nucleotide conserved sequence near the 5' end is also thought to be the complement of the sequence involved in the initiation of RNA^- synthesis (see below) (Ou et al., 1983). A 21 nucleotide sequence has been found at the junction between the 26S RNA (2 nucleotides) and the non-structural polypeptide coding sequence (19 nucleotides) and is thought to be the initiation sequence on the 42S RNA^- strand for transcription of 26S RNA (Ou et al., 1982a; Reidel et al., 1982) (also see Fig. 3). The sequences of the 3'

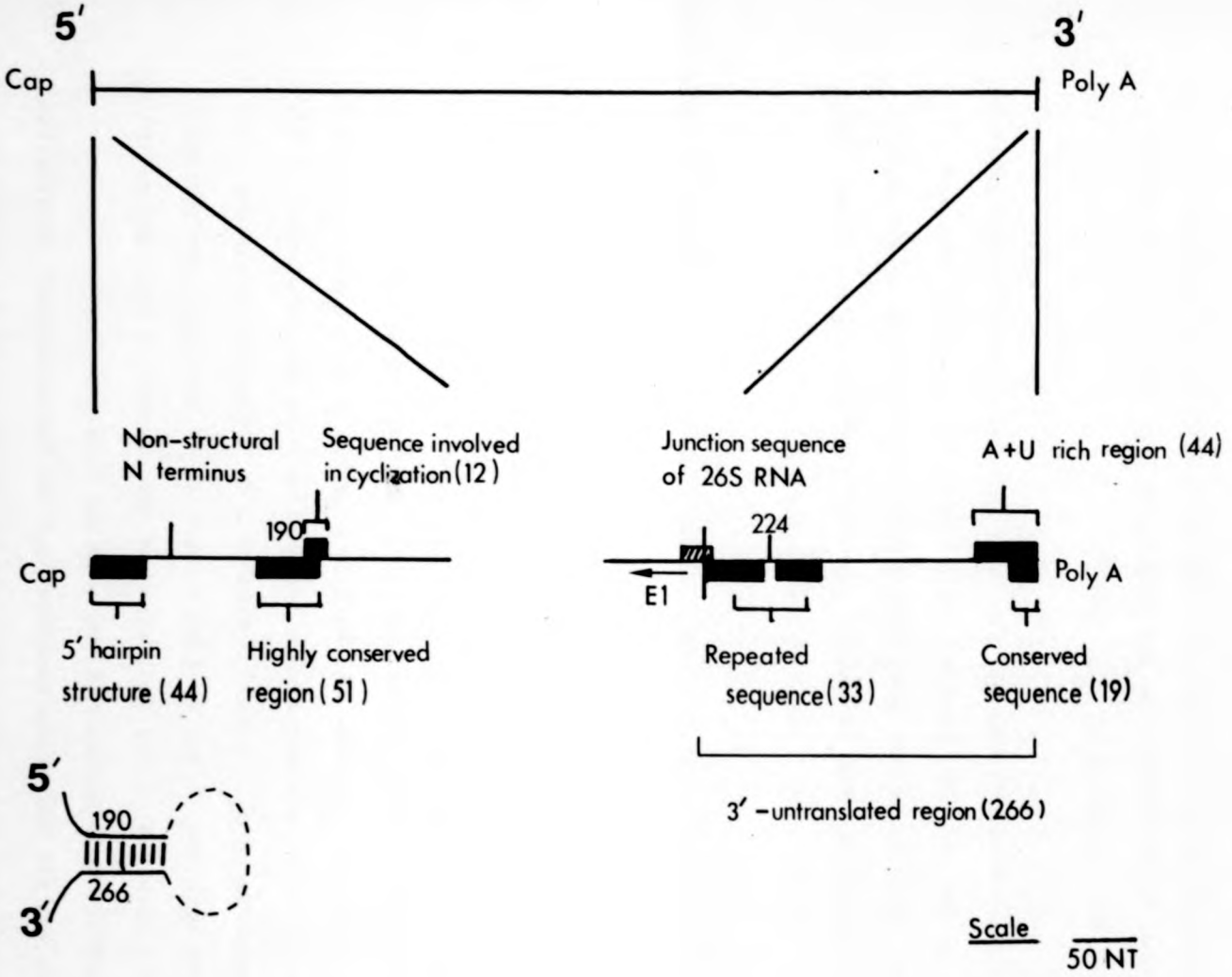

5′
3′
Cap
Poly A
Non-structural
N terminus
Sequence involved
in cyclization (12)
190
Cap
5′ hairpin
structure (44)
Highly conserved
region (51)
Junction sequence
of 26S RNA
A+U rich region (44)
224
E1
Poly A
Repeated
sequence (33)
Conserved
sequence (19)
3′ -untranslated region (266)
5′
190
266
3′
Scale
50 NT

Figure 3. Sequence and structure of alphavirus genomes

(adapted from Ou et al., 1982b, 1983). Numbers refer to nucleotides and are numbered from the 5' or 3' terminus as appropriate.

end (19 nucleotides) and the junction (21 nucleotides) control elements are different, which presumably allows differential regulation of RNA synthesis.

At the 5' terminus, a 44 nucleotide sequence is present which can be formed into a stem and loop structure while about 150 nucleotides from the 5' end a 51 nucleotide sequence (see below) is found which contains complementary sequences such that two stable hairpin structures can be formed. It is thought that these structures on the RNA^- strand may be involved in the initiation of RNA^+ synthesis (Ou et al., 1983). Within the 266 nucleotide non-coding region, present at the 3' end of the genome (Garoff et al., 1980b; Ou et al., 1982b), the first 50 nucleotides adjacent to the 3' terminal poly A tract are highly A + U rich (80%) and it is thought that this sequence may be a signal for polyadenylation (Ou et al., 1982b). Two 33 nucleotide repeat sequences have also been found in the 3' non-coding region but the significance of these is not understood at present (Ou et al., 1982b).

Earlier studies by Hsu et al. (1973) and Frey et al. (1979) suggested that alphavirus RNAs are able to cyclise and form panhandle structures. From sequence studies, Ou et al. (1983) have suggested that a panhandle structure is possible using part of the 51 nucleotide sequence at the 5' end of the RNA and complementary sequences near the 3' end of the genome. In this model both 3' and 5' termini are not present in the stem of the panhandle. It should be noted that Ou et al. (1983) were unable to match sequences perfectly, and so this model should be interpreted with caution. The features of the sequence studies are

summarised in Figure 3. The sequence data discussed above will become important when compared to the sequence content of DI RNA species (see later).

vi) Translation of structural proteins

The subgenomic 26S RNA codes for all the structural proteins and these are translated from a single initiation site (Clegg and Kennedy, 1975). The proteins are synthesised sequentially as a large precursor polypeptide of M_r 130,000 (Clegg, 1975; Clegg and Kennedy, 1975) and the individual polypeptides are formed by post translation cleavages of the nascent precursor (see Fig. 4).

Recently, a small non-structural polypeptide (M_r 6,000) has been found to be encoded by the 26S RNA and it is thought that it serves as a recognition sequence for cleavage of E1 (Welch and Sefton, 1979, 1980; Garoff et al., 1980b; Hashimoto et al., 1981). The C polypeptide is cleaved from the nascent precursor immediately after it is completed and associates with the progeny genomes to form nucleocapsids in the cell cytoplasm (Garoff et al., 1978). After the cleavage of C polypeptide on free ribosomes, the ribosomes move to the rough endoplasmic reticulum where the synthesis of the envelope polypeptides takes place. This is accompanied by membrane translocation and glycosylation (Garoff et al., 1978). The 26S mRNA is unusual in directing the synthesis of proteins which are distributed to different parts of the cell, i.e. the C polypeptide into the cytoplasm and envelope polypeptides into the endoplasmic reticulum.

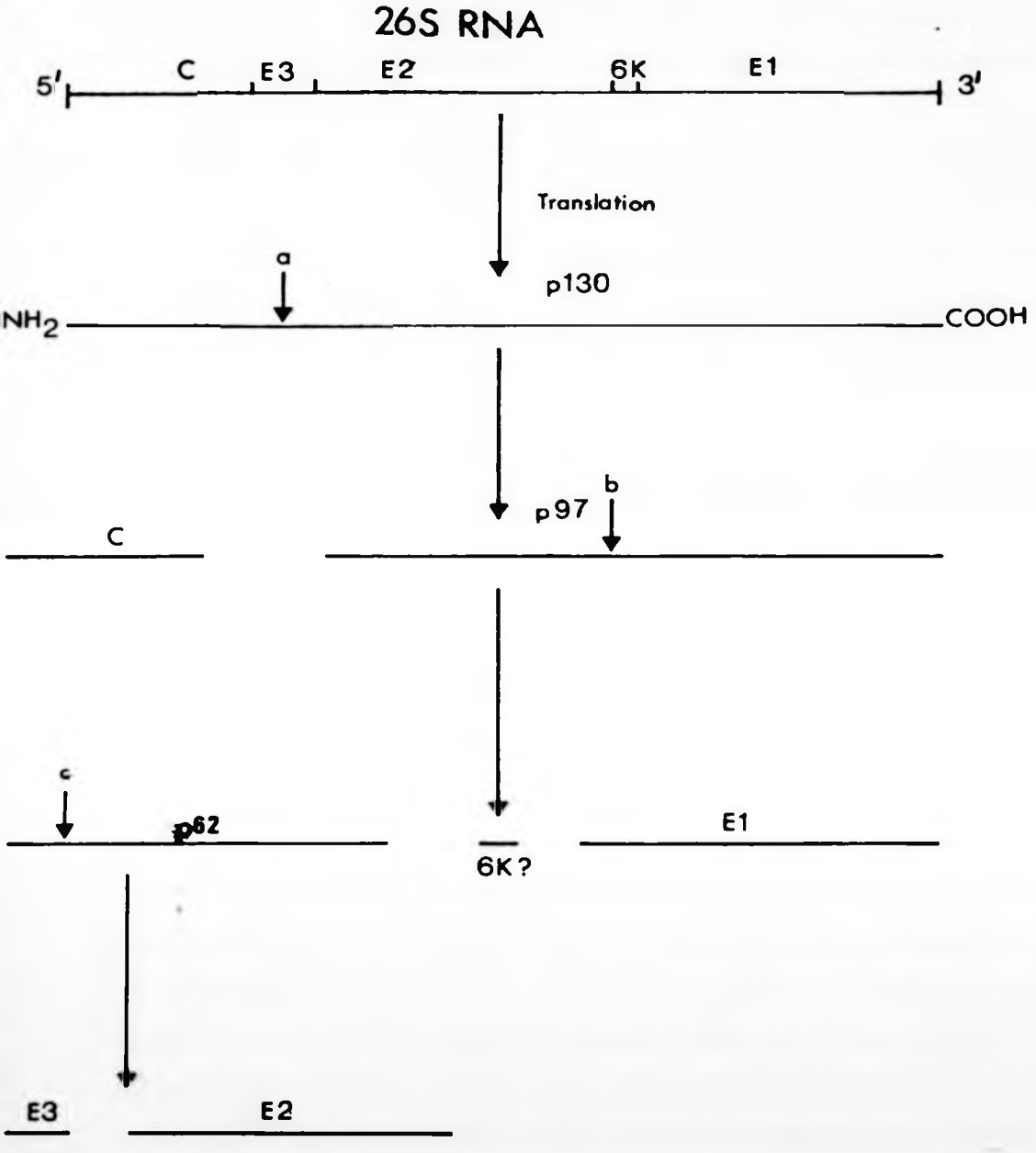

26S RNA
5'
C
E3
E2
6K
E1
3'
Translation
a
p130
NH2
COOH
b
p97
C
c
p62
6K?
E1
E3
E2

Figure 4. Post-translational cleavages in the formation of the structural proteins of SFV

The cleavage of 6K has not yet been characterised and its position in the cleavage pattern is speculative, and is assumed from its position in the sequence of the 26S RNA.

vii) Assembly

The way in which C protein binds to the 42S RNA^+ is unknown at present, but Wengler et al. (1982a), using an in vitro system, have suggested that assembly of nucleocapsid depends upon the relative concentrations of protein and RNA available. Only 42S RNA^+, and not 26S RNA^+, is encapsidated (Ulmanen et al., 1976), therefore the 5' two-thirds of the genome probably has a specific binding site for C protein.

The newly synthesised p62 and E1 glycopolypeptides form a complex which is transported through the Golgi complex to the plasma membrane of the host cell. Here p62 is cleaved to form E2 and E3. The glycopolypeptides are inserted into the plasma membrane by fusion of the transport vesicles with the cell surface (Kääriäinen and Renkonen, 1977; Ziemiecki et al., 1980; Garoff et al., 1982).

Electron microscopy has shown that the nucleocapsid attaches to the cytoplasmic side of the modified plasma membrane, which then begins to form a bud partly surrounding the nucleocapsid (Acheson and Tamm, 1967; Erlandson et al., 1969; Bykovksy et al., 1969; Grimley and Friedman, 1970; Grimley et al., 1972; Brown et al., 1972). This process probably is helped by the nucleocapsid attaching to the cytoplasmic side of the transmembrane portion of the virus spike (Garoff and Simmons, 1974). Since E2 is thought to span the membrane to attach to C protein; membrane is able to engulf the nucleocapsid by the E2 portion of the spike binding to sites present on the C protein of the nucleocapsid. Once all the binding sites are occupied and the nucleocapsid is completely surrounded by the membrane, the bud finally breaks off thus

producing a progeny virus particle. In mosquito cells it is thought that virus assembles at intracellular membranes, budding into vacuoles which release the virus particles into the extracellular environment by fusing with the plasma membrane (Whitefield et al., 1971, Raghow et al., 1973; Gliedman et al., 1975).

2. DEFECTIVE INTERFERING VIRUSES

a) Glossary

The following terms are commonly used when discussing defective interfering viruses:

Standard virus (S virus) is an infectious virus preparation.

Defective interfering virus (DI virus) has part of the S virus genome deleted and is thus non-infectious. Consequently, propagation requires the presence of S virus and this results in interference with the multiplication of S virus.

Co-infection is when a cell is infected by DI virus and S virus.

DI virus preparations are the stocks of DI virus grown in tissue culture for use in the experiments described below. They contain S virus (usually $<10^7$ pfu/ml) in addition to DI virus.

Serial undiluted passage is the method used for the propagation of DI virus preparations. The progeny from one passage is used as the inoculum for the next passage while maintaining a moi of 50 by the addition of S virus as necessary.

Passage (p) number refers to the number (X) of serial undiluted passages, (pX).

Interference occurs when co-infection of cells results in the DI virus competing with the S virus for a limited component of the virus multiplication cycle. This results in the replication of DI virus at the expense of S virus, thus the term interference. Interference is usually specific for the parental S virus and is referred to as homologous interference.

Generation are the initial events which result in the loss of S genome sequence and the production of DI virus.

Enrichment is the phenomenon by which DI particles increase in a population, usually after serial undiluted passage.

b) Historical overview

In 1947 von Magnus reported that non-infectious virus appeared when undiluted influenza virus was passaged by allantoic inoculation of embryonated chicken eggs. Successive passages resulted in a decrease in the ratio of virus infectivity to haemagglutinin (von Magnus, 1951a, b,

1954). He termed the non-infectious virus "incomplete". Co-infection of eggs with "late" passage (incomplete virus) and "early" passage virus resulted in interference with the multiplication of "early" passage virus. However, if the "late" passage virus was diluted before being used as inoculum for the next passage, the ratio of infectivity to haemagglutinin remained high and no interference was detected.

In the 10 years following von Magnus' observations little work was published on the interference phenomenon but in 1959, Bellett and Cooper first demonstrated the interference phenomenon in tissue culture with vesicular stomatitis virus (VSV). From 1959 onwards, as the techniques of molecular biology rapidly increased, so did the studies on the phenomenon of interference. In 1970 Huang and Baltimore defined defective interfering (DI) particles as the cause of interference and described key properties of these particles in order to distinguish this type of interference from other types. The properties of DI particles are shown in Table 1 and all originate from the fact that DI particles possess a genome derived by a process of deletion from standard virus. Huang and Baltimore went on to speculate that DI viruses may play a role in the modulation of acute and persistent virus infections in nature.

c) Nomenclature

To date a vast literature has been accumulated on DI particles which has used a variety of terms to describe DI and standard virus:

DI virus particles have been termed non-infectious, von Magnus, auto-

Table 1 Properties of DI particles

1. Have a genome derived by deletion from the genome of the S virus.

2. Use the S virus structural proteins and hence are antigenically identical.

3. Require the presence of S virus for replication. Consequently, DI particles cannot propagate unaided. Thus the virus is termed DEFECTIVE.

4. Reduce the yield of S virus from the progeny of the co-infected cells. Thus the virus INTERFERES.

5. Interfere specifically with the homologous S virus.

6. During co-infection DI virus interferes with an intracellular step of the growth cycle of the S virus.

7. Must possess a "functional" nucleic acid, i.e. interference is not due to a protein or "soluble factors".

8. Interferon plays no role in the interference phenomenon, although DI particles themselves may induce interferon.

9. Prior UV irradiation of the DI particles destroys the interference phenomenon.

interfering, incomplete, defective, interfering, deleted or immature virus. Many of these terms are used interchangeably and are ambiguous and do not fully describe the properties of these viruses. Therefore, this thesis will use the term defective interfering (DI) virus particles exclusively. For standard virus the terms, wild-type, ts^+, complete, parental, normal, non-defective, competent or helper virus have been used. In this thesis the term standard (S) virus will be used.

d) Interference and DI particles

At least three types of virus interference have been described: interference by unconditional defective (DI) particles, conditional defective particles (temperature sensitive mutants) (Youngner and Quaglina, 1976) or non-defective heterotypic virus (Legault et al., 1977) and very little is known about the mechanisms involved in any of these three types of interference. It should be emphasised here that serial high multiplicity passage results in the formation of many types of genetic variant besides DI particles, including temperature sensitive mutants, which have also been found to be capable of producing interference (Stollar et al., 1973; Youngner and Quaglina, 1976; Keränen, 1977; Chakraborty et al., 1979; King et al., 1979). In particular, work with reovirus has shown that serial high multiplicity passage results in the formation of DI particles, temperature sensitive mutants and growth attenuated mutants (Ahmed et al., 1980; Ahmed and Fields, 1981). The latter were found to be small plaque/low yield mutants which could be rescued by mixed infection with S virus. Non-interfering and interfering defective particles (Frenkel et al., 1975;

Kawai and Matsumoto, 1977) and infectious virus resistant to DI particle-mediated interference (Jacobson and Pfau, 1980; Horodyski and Holland, 1980; Weiss and Schlesinger; 1981; Brinton and Fernandez, 1983) have also been reported. However, the latter have only been isolated from persistent infections and are thought to represent the continuous and extensive mutational changes or "evolution of viruses" involved in these types of infections (for a review see Holland et al., 1982).

DI particles and/or interference phenomena have been observed in almost every group of animal viruses grown in tissue culture (Huang and Baltimore, 1977; Perrault, 1981) and although DI particles have not been characterised in each system, it seems probable that DI particles are ubiquitous. DI particles of plant viruses (Huang, 1973) and bacteriophage (Enea and Zinder, 1975) have also been described.

e) Properties of DI particles

Since the discovery of DI particles, a vast literature on the subject has developed. Many virus systems have been shown to have DI particles with properties unique to that virus group, and a variety of interference mechanisms are evident. Consequently reviews on DI particles are now appearing on a single virus group rather than on the whole subject (see Table 2), although the review by Perrault (1981) provides an excellent, but unfortunately already dated, survey of DI particles. With this in mind, a review of all DI particles would be beyond the scope of this thesis, so the discussion of DI particles will

Table 2 Recent reviews of DI particles from various virus groups

Subject	Reference
General	Huang and Baltimore, 1970, 1977; Huang, 1973.
RNA viruses	Holland et al., 1980; Lazzarini, et al., 1981; Perrault, 1981.
Alphaviruses	Stollar, 1979, 1980.
Arenaviruses	Pfau, 1977.
Herpesviruses	Frenkel, 1980.
Influenza viruses	von Magnus, 1954; Nayak, 1980.
Poliovirus	Cole, 1975.
Rhabdoviruses	Reichmann and Schnitzlein, 1979; Huang, 1982.

centre on alphaviruses and, where appropriate, comparisons will be made with other virus systems.

f) DI alphaviruses

DI alphaviruses were first reported for Sindbis virus (SV) by Schlesinger et al. (1972) and Inglot et al. (1973) and later by Bruton and Kennedy (1976) for SFV and Martin et al. (1979) for Ross River virus. To date these are the only alphavirus DI particles described. Like other DI viruses, DI alphaviruses arise on serial undiluted passage and reduce the yield of S virus (Schlesinger et al., 1972; Bruton and Kennedy, 1976). Virus purified from low multiplicity passage was found to contain only 42S standard virus RNA, while virus obtained after serial undiluted passage contained 2 or more species of RNA. Although some 42S RNA was observed, the majority was found to be smaller than the genomic RNA. It was suggested that these were DI RNA species (Shenk and Stollar, 1973a; Weiss and Schlesinger, 1973; Johnston et al., 1975; Bruton and Kennedy, 1976).

i) Separation of DI particles

Attempts at separating alphavirus DI and S virus particles suggests that there is little difference in size and density between the two particles. Early experiments by Shenk and Stollar (1973b) reported that DI SV could be physically separated from S virus, while later work by the same group (Guild and Stollar, 1975) failed to separate the two particles. Weiss and Schlesinger (1973) have also failed to separate DI

SV from S virus. Bruton and Kennedy (1976) have reported that they could separate DI SFV from S virus by caesium chloride density gradient centrifugation but this destroys biological activity. Logan (1979) and Kääriäinen et al. (1981) failed to separate DI SFV by sucrose gradient centrifugation. Centrifugation in caesium chloride indicates that DI particles are more dense (1.22 to 1.23 gml^{-1}) than S virus (1.20 gml^{-1}) (Shenk and Stollar, 1973a; Bruton and Kennedy, 1976) but Shenk and Stollar (1973b) found interfering activity across the gradient from densities 1.20 to 1.22 gml^{-1}. This lack of reproducibility has made studies of DI particles difficult. To overcome this problem research into DI alphaviruses has centred on the events which occur in co-infected cells.

ii) Alterations in the SFV replication cycle in co-infected cultures

A number of changes have been reported in co-infected cultures. All workers have reported an alteration in the viral RNA species synthesised in co-infected cultures. A large reduction in the 42S and 26S RNA of S virus is observed with the concomitant appearance of sub-genomic viral RNA species, which are the DI RNA species (Shenk and Stollar, 1972; Eaton and Faulkner, 1973; Levin et al., 1973; Weiss and Schlesinger, 1973; Weiss et al., 1974; Eaton, 1975; Guild and Stollar, 1975; Igarashi and Stollar, 1976; Bruton et al., 1976; Logan, 1979). Levin et al. (1973) have reported a reduction in the number of membraneous structures associated with the replication complexes compared with S virus infected cells. Weiss et al. (1974) found that DI RNA was polyadenylated but would not function as a mRNA in vitro and only

functioned poorly *in vivo* and did not associate with ribosomes. Bruton *et al.* (1976) took purified DI SFV and found it was unable to carry out any part of the virus replication cycle other than uncoating. It was also unable to function as a mRNA *in vivo* or *in vitro*. However, Weiss and Schlesinger (1973) observed a new polypeptide of M_r 75,000 in cells co-infected with DI SV. Similarly, Guild and Stollar (1975) have reported a polypeptide of M_r 80,000 in DI SV co-infected cells. These studies would suggest that DI RNA or some DI RNAs can act as functional mRNAs. Fuller and Marcus (1980a, b) have suggested that early passage (p5) DI SV can be translated while late passage (p15) virus cannot, since p5 DI SV could induce interferon production while p15 DI SV could not. Although this may be true, the results of Fuller and Marcus (1980a, b) are open to question because it is unclear if translation was obtained from DI virus or contaminating S virus. However, if true these results may explain the difference in reported results since if the deletion of the DI RNA is small it is possible that the 5' initiation site of the 42S RNA may be retained in the DI RNA so that translation can occur. Conversely if the deletion is large the sequences necessary for initiation of translation may be deleted.

While the translational ability of DI RNA has still to be clarified there is agreement that alphavirus DI RNA is not transcribed or replicated in the absence of S virus (Shenk and Stollar, 1973a; Weiss et al., 1974; Bruton and Kennedy, 1976). Interference is thought to occur at the level of virus-specified RNA synthesis, since the synthesis of DI RNA takes place at the expense of S virus RNA species (see above). Inhibition of both S virus RNA^+ and RNA^- is observed as well as the

synthesis of double-stranded replicative intermediates (Shenk and Stollar, 1972; Eaton and Faulkner, 1973; Kennedy et al., 1976; Bruton and Kennedy, 1976; Guild and Stollar, 1975; Guild et al., 1977). Interference results in a reduction in the amount of total viral RNA synthesis (Guild and Stollar, 1975; Barrett et al., 1981) and this is dependent on the ratio of DI to S virus in the inoculum. This taken together with the results described above, is thought to explain the reduction in synthesis of S virus polypeptides in co-infected cells (Weiss and Schlesinger, 1973; Bruton et al., 1976; Logan, 1979). Bruton et al. (1976) have observed that DI virus may interfere with S virus in co-infected cells so much that no virus polypeptides are synthesised and no shut off of host protein synthesis is observed.

iii) Properties and replication of alphavirus DI RNAs

Hybridisation studies (Weiss et al., 1974; Bruton et al., 1976; Guild and Stollar, 1977; Guild et al., 1977) and T1 oligonucleotide mapping (Kennedy, 1976; Kennedy et al., 1976; Stark and Kennedy, 1978) have led to the conclusion that all DI genomes retain some sequences from both the 3' and 5' ends of the S virus genome. Therefore, all deletions of alphavirus genomes are internal. These experiments also showed that DI virus RNAs are polyadenylated, positive sense (i.e. RNA^+) and contain only virus-coded sequences. Retention of the termini are thought to be due to the necessity to retain the initiation sites for RNA replication of both RNA^- and RNA^+. In the S virus multiplication cycle only 42S RNA^+ is encapsidated, and not 26S RNA^+ or 42S RNA^-, thus the capsid binding site must be located near one of the termini to enable progeny

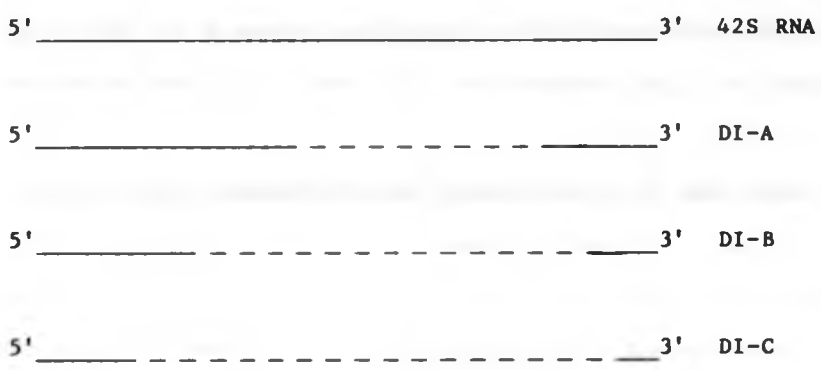
DI particles to be produced. Synthesis of DI RNA has been shown not to involve 42S RNA$^-$ (Bruton et al., 1976; Brzeski and Kennedy, 1978), and RFs and RIs equivalent in size to DI RNAs have been detected in co-infected cells (Eaton and Faulkner, 1973; Weiss et al., 1974; Kennedy et al., 1976; Bruton et al., 1976; Guild et al., 1977; Guild and Stollar, 1977). It is generally agreed that replication of DI RNA is similar to that of S virus requiring S virus RNA synthesizing enzymes, but is independent of any S virus RNA species except that the 42S RNA codes for all the viral proteins, both structural and non-structural.

iv) Interference, propagation and generation of alphavirus DI RNA

In 1975 Johnston et al. observed that SV DI RNA decreased in size during serial undiluted passage. Similar results were obtained by Guild et al. (1977) for SV and Stark and Kennedy (1978) for SFV. Since the S virus termini were retained in the DI RNA a model was proposed to explain the sequence organisation of alphavirus DI RNAs by Kennedy (1976) and Stark and Kennedy (1978) for SFV (Fig. 5) and Guild and Stollar (1977) for SV. It was proposed that the larger DI RNAs were the progenitors of shorter RNAs. For example in Fig.5, serial undiluted passage would produce particles containing DI-A, which upon further passaging would give rise to particles containing DI-B with the concomitant disappearance of those containing DI-A. By a similar argument DI-C will replace DI-B. DI-C will contain all the sequences in DI-B, and all sequences in DI-B will be present in DI-A. Thus passaging will result in bigger internal deletions and smaller DI RNAs.

Figure 5. Proposed sequence organisation of SFV DI RNAs

Solid lines represent conserved regions of the standard virus growth and dashed lines deleted portions. Adapted from Stark and Kennedy (1978).



Since the 3' end of DI and S virus RNA is identical it was proposed that interference took place by competition between the two RNA species for available polymerase. Assuming that polymerase carries out synthesis of DI and S virus RNA^+ templates at an identical rate, more of the shorter DI RNA^- will be synthesised in a given period of time. Also since the 5' ends of DI and S virus RNA are identical, DI and S virus RNA^- will compete for available polymerase which will result in greater synthesis of DI RNA^+ by a manner analogous to that described above. By a similar mechanism smaller DI RNAs will out-compete larger DI RNAs.

It was further suggested that generation of DI RNA takes place by a recombinational event during transcription. Hsu *et al*. (1974) and Frey *et al*. (1979) have shown that SV 42S RNA forms circles whose ends form a panhandle structure. The panhandle occurs because the 5' and 3' ends of the RNA are inverted complements of each other (Ou *et al*., 1983). It is thought that the polymerase detaches from the template and then rejoins it at a point nearer the 5' end, thus deleting the RNA. It is proposed that this "jumping" takes place due to the secondary structure of the RNA where the "detaching" and "rejoining" points are adjacent to each other. Once generated, the DI RNA can then interfere with the S virus RNA species.

v) Sequence and structure of DI RNA

Until recently, the models for interference and generation described above were not disputed. However, using T_1 oligonucleotide mapping Stark and Kennedy (1978) reported one SFV DI RNA which had two

deletions, while Dohner et al. (1979) described a SV DI RNA with multiple deletions. The latter workers also observed that some oligonucleotides were present in non-equimolar amounts suggesting that some sequences may be present in more than one copy. Further T_1 oligonucleotide mapping studies by Pettersson (1981) of a p11 DI SFV 18S RNA showed that the oligonucleotides were clearly non-equimolar (in contradiction to all previous studies, except that of Dohner et al., 1979).

Pettersson also found that the 5' terminal cap sequences were different from S virus 42S or 26S RNA and were heterogeneous:

42S RNA	7mGpppAUG	
26S RNA	7mGpppAUUG	
18S DI RNA	7mGpppAU$(AU)_n$CAUG	n = 4-8

Pettersson (1981) proposed that the extreme 5' terminus of 42S RNA was not conserved in the DI RNAs and that there was heterogeneity in the 18S DI RNA population. These studies were continued by Kääriäinen et al. (1981), who described the appearance of DI RNA species during serial undiluted passage. An 18S RNA appeared at p4 and remained the predominant RNA species until p17 when a 24S RNA species, which had been present in small amounts up to p17, became predominant. A 33S RNA species appeared at p21. This increase in size of DI RNA during passage contrasted with the observations of Kennedy (1976), Stark and Kennedy (1978) and Guild and Stollar (1977) who proposed a decrease in size of DI RNA during passaging. Kääriäinen et al. (1981) showed that although

the 18S DI RNA was replicated rapidly, it was inefficiently encapsidated into progeny virus particles, while the small quantities of 24S DI RNA, present upto p17, were replicated poorly but encapsidated 3 to 5 times more efficiently than the 18S RNA. T_1 oligonucleotide mapping studies showed that the early passage and late passage 24S DI RNAs were related, but not identical, while the early passage 24S DI RNA had a T_1 map similar to 18S DI RNA. Late passage 24S DI RNA had a T_1 fingerprint identical to 33S DI RNA. Since all the T_1 maps had oligonucleotides in non-equimolar quantities it was proposed that the 18S DI RNA population was heterogeneous and contained repeat sequences which were present in greater numbers (5 to 10 copies) in the 24S and 33S DI RNAs.

The nucleotide sequence of DI RNAs was next determined from cloned DNA copies and this will now be compared to the sequences present in S virus RNA (see Section 1.f.v). Different clones of the p11 18S DI RNA were found to have different sequences and each sequence contained repeat units (Söderlund *et al.*, 1981; Lehtovaara *et al.*, 1981, 1982). Two clones, pKTH301 (Lehtovaara *et al.*, 1981) and pKTH309 (Lehtovaara *et al.*, 1982) were examined in detail (see Fig.6). pKTH301 was a major and pKTH309 a minor DI RNA species in the 18S DI RNA population. pKTH301 contained 3 repeat units of 484 nucleotides of which the 3' terminal repeat has a 60 nucleotide "insert". pKTH309 consists of a duplicated region with flanking unique terminal sequences and 4 repeat units of 273 nucleotides. The repeat units of the two clones were not the same. The 3' end of the DI RNAs were all identical to that of the S virus (pKTH301, 302, 306 and 309 had 106, 84, 106 and 102 nucleotides respectively, identical to S virus). This region is very A-T rich

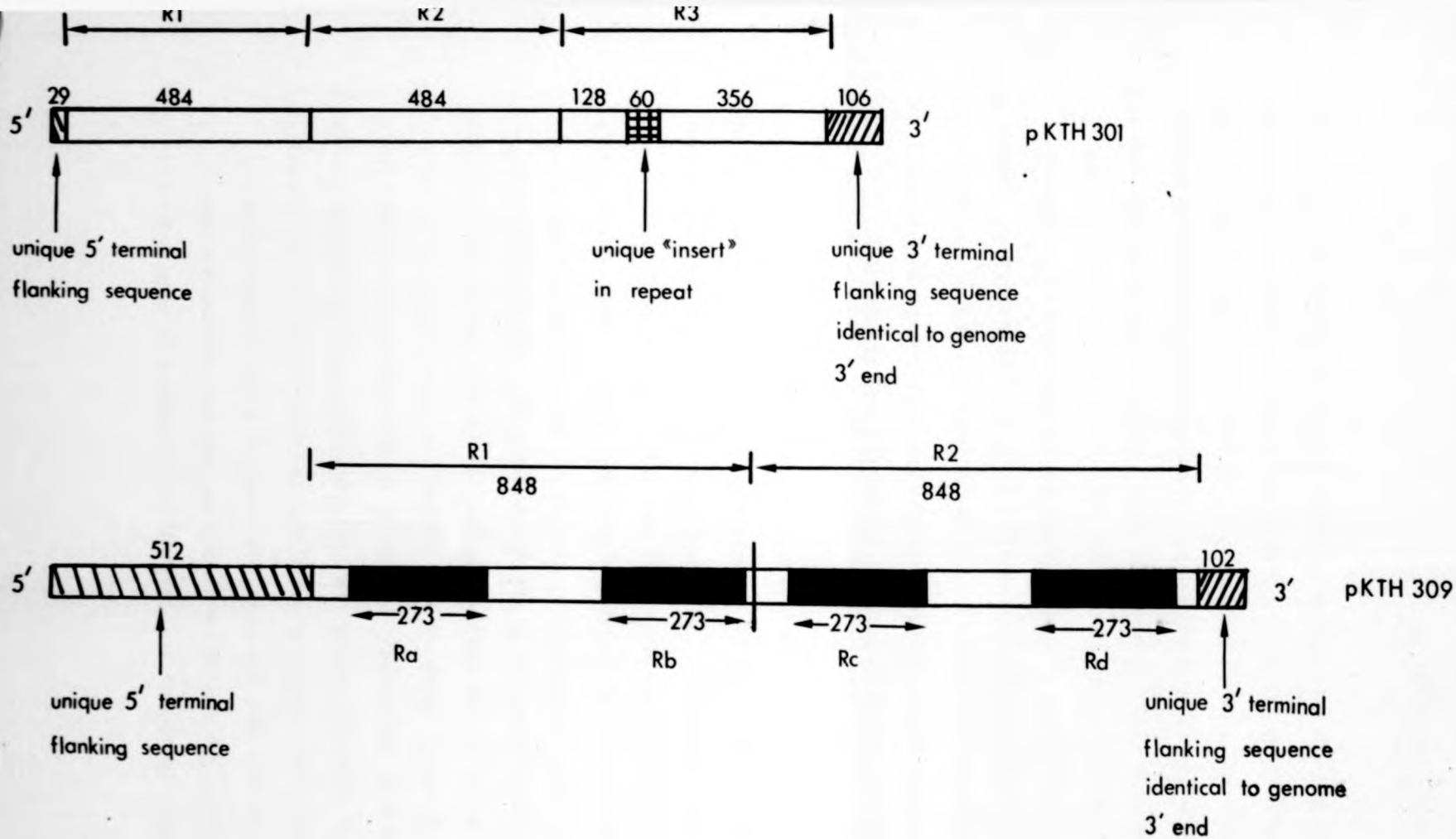

R1
R2
R3
29
484
484
128
60
356
106
5′
3′
pKTH 301
unique 5′ terminal flanking sequence
unique «insert» in repeat
unique 3′ terminal flanking sequence identical to genome 3′ end
R1
848
R2
848
512
102
5′
3′
pKTH 309
273
273
273
273
Ra
Rb
Rc
Rd
unique 5′ terminal flanking sequence
unique 3′ terminal flanking sequence identical to genome 3′ end

Figure 6. Diagrammatic representation of the structure of complementary DNA copies of two SFV DI RNAs

Adapted from Lehtovaara et al. (1981, 1982). Sequences of DI RNAs from two clones, pKTH301 and pKTH309, obtained from the same DI virus preparation are shown. The repeat units in each clone are referred to a "R". DI RNA pKTH309 contains two tandemly joined repeat units, R1 and R2 of which each contains two smaller repeats, Ra and Rb, and Rc and Rd respectively. Unique refers to sequences which are present in S virus RNA but have been rearranged (see text).

(75%), as found for S virus, and is thought to be involved in polymerase binding. However, the 3' end of 42S RNA is identical to 26S RNA and the latter is not replicated, therefore additional sequences must be involved in replication. Although the 3' terminal sequence is conserved in each DI RNA, the adjoining sequences differed for each DI RNA studied. Thus the sequences involved in replication remain to be elucidated. The extreme 5' terminus of S virus has also been retained in DI RNA but in pKTH309 this sequence has been deleted, rearranged and duplicated. Only 29 nucleotides are present in the 5' terminus unique region of pKTH301 because cloning failed to include the very 5' terminal sequence (approximately 200 nucleotides). The 5' terminal conserved 47 nucleotide region in S virus is retained in DI RNA and is present in 1 copy for pKTH309 but 3 copies for pKTH301 (1 in each repeat unit). It is thought this region is involved in polymerase recognition. In pKT301 a 278 nucleotide element has been translocated from the 5' terminus (nucleotides 39 to 317) to approximately 84 nucleotides from the 3' end and the sequence thought to be involved in cyclization has been removed (Lehtovaara et al., 1981). Whether this gives DI RNA a replicative advantage is unknown, but no cyclization could result in less time and energy required for replication.

Recently sequence studies have also been carried out on SV DI RNA. The results obtained were similar to SFV. DI RNA was heterogeneous in size and contained multiple deletions and sequence rearrangements (Monroe et al., 1982). Both DI RNAs examined retained the 3' end 50 nucleotides of the S virus including the 20 nucleotide region regulatory element involved in replication. As observed for DI SFV (Pettersson, 1981), the

5' terminus cap structure has a different sequence to S virus (Ou et al., 1983):

DI RNA ^{7}mGpppAXAXA

42S RNA ^{7}mGpppAUUG

but the 5' end 51 nucleotide conserved region present in S virus is present in SV DI RNA (Monroe et al., 1982) further suggesting that this sequence is involved in replication. The regulatory element in SV S virus RNA, which is approximately 240 nucleotides from the 3' terminus, is not retained in SV DI RNA (Monroe et al., 1982). Therefore it would appear that this sequence is not essential for replication or packaging of RNA genomes. Very recently, Monroe and Schlesinger (1983) have determined the 5' terminal sequence of 2 DI RNAs and found that they are identical to cellular tRNA^{ASP} and thus are not of viral origin. The significance of this sequence is unknown at present.

From the above it is clear that some, but not all, regulatory elements of S virus have been retained by DI RNA. The mechanism of generation of DI RNA would appear to involve both a deletion mechanism and an amplification mechanism and cannot be simply explained by internal deletion as previously proposed (Kennedy, 1976; Stark and Kennedy, 1978; Guild and Stollar, 1977). How the repeat sequence units are formed is unknown but Ou et al. (1982b) have proposed that a template switching mechanism may take place and the short preceeding sequence may aid template switching, due to the retention of the S virus polymerase binding site at the 3' terminus of DI RNA.

Repeat units in DI genomes are not unique to alphaviruses since they have also been found in papovaviruses, herpes viruses and phages (Fareed and Davoli, 1977; Ravetch et al., 1979; Schaller, 1979). Whether this represents a common strategy for generation and interference by DI viruses remains to be seen. To date, influenza virus appears to have DI particles most closely related to those of alphaviruses. Studies on the sequences of influenza DI virus genomes have shown that some DI RNAs are made up of a mosaic of sequence rearrangements from segments 1 and 3 (Fields and Winter, 1982), while Nayak et al. have shown that DI influenza virus contains both single and multiple internal deletions (Davis and Nayak, 1979; Davis et al., 1980; Nayak et al., 1982; Sivasubramanian and Nayak, 1983).

vi.) Packaging of DI RNAs

Since it has proved difficult to separate DI particles from S virus it has been proposed that DI particles contain approximately the same total amount of RNA as the S virus genome, i.e. 4.3×10^6 (Kennedy et al., 1976; Guild et al., 1977). Therefore each DI particle contains several molecules of DI RNA (Bruton and Kennedy, 1976; Kennedy et al., 1976; Guild et al., 1977). Since Bruton and Kennedy (1976) have managed to separate DI and S virus particles they propose that each particle contains more RNA per particle than S virus particles. However, this does not explain the single hit UV inactivation kinetics of DI virus (Kowal and Stollar, 1980; Barrett et al., 1981). Overall it would appear that there are factors (unknown at present) which control the packaging and production of progeny DI virus particles. This was

confirmed by Wengler et al. (1982a) who have developed an in vitro nucleocapsid assembly system which has been found to have constraints on nucleocapsid size.

vii) Effect of host cell upon generation and replication of DI particles

Levin et al. (1973) were the first to observe the effect of the host cell upon the generation and replication of alphavirus DI particles. Later, a detailed study by Stark and Kennedy (1978) showed that some cell types (e.g. mouse 3T3 and rat NRK) generated and enriched DI particles very rapidly, whereas others took many passages to generate DI particles (e.g. HeLa, 20 passages). Recently, Barrett et al. (1981) showed that DI SFV differed in its ability to interfere with S virus depending upon the cell type used. The reason for the difference between cell types is unknown but may be due to the host-coded components of the virus polymerase (Clewley and Kennedy, 1976) or to the structure of the replication complex in different cell types.

Since alphaviruses replicate in mosquito cells, the ability of these cells to support the propagation of DI particles has been examined. Both SFV and SV DI particles have been shown to be generated and enriched in mosquito cells. Early work showed that DI particles generated in vertebrate cells would not replicate in mosquito cells (Igarashi and Stollar, 1976; Eaton, 1975; King et al., 1979), however Logan (1979) and Tooker and Kennedy (1981) have generated DI particles in the first passage in cloned mosquito cells using S virus grown in vertebrate cells. Logan (1979) showed that DI SFV generated in mosquito cells

interfered with the multiplication of S virus in vertebrate CEF cells. Tooker and Kennedy (1981) have T_1 oligonucleotide mapped DI SFV generated in mosquito cells and found it has an essentially identical map to that generated in vertebrate cells, suggesting that DI RNA is generated in vertebrate and mosquito cells by a similar mechanism. However, Kowal and Stollar (1980) generated DI SV in BHK and mosquito cells and sized the RNA on agarose gels. The UV target size of BHK cell derived DI SV correlated well with the physical size of the genome, while the UV target size for mosquito cell derived DI SV was 25-30% smaller than the physical size of the genome. This would suggest that the whole genome is required for interference in BHK cells but only part is required in mosquito cells.

viii) Ross River virus

As stated earlier, DI particles have only been reported for 3 alphaviruses, SFV, SV and Ross River virus. In comparison to extensive studies of SFV and SV only one report has appeared on DI Ross River virus (Martin et al., 1979) and this proposes that DI particles interfere at the level of translation. This is clearly in contrast to results obtained for SFV and SV where interference is thought to take place at the level of RNA synthesis. If true, it would appear that DI Ross River virus interferes in a manner analagous to poliovirus (see Cole, 1975) and emphasises the point that generalizations about DI viruses should be made with great care.

g) Persistent infections

Persistent infections of both tissue culture cells and the whole animal have been described for a number of viruses including RNA viruses (for a review see Holland et al., 1980). For a persistant infection to be established an individual host cell or a proportion of the population must survive infection, i.e. there is a balance between virus multiplication and death of cells. In tissue culture the cells often go through a period of "crisis" where most cells die, but a few survive to continue growing. Normally virus mutants appear which maintain the persistent infection and have reduced cytopathogenicity. They produce low levels of virus progeny and the cell population continues to grow. A number of factors have been implicated in the establishment and maintenance of persistent infection including mutation of standard virus (particularly temperature sensitive mutants), interferon production, integrated DNA copies of RNA viruses, and DI particles. These have been extensively reviewed in Friedman and Ramseur (1979), Persistent Viruses (1979) and Holland et al. (1980, 1982).

Huang and Baltimore (1970) were the first to propose a role for DI particles in the establishment and maintenance of persistent infections and since 1970 a number of workers have reported a role for DI particles in a number of virus systems for modulating persistent infections (for a review see Holland et al., 1980).

Standard alphaviruses are normally lethal for vertebrate cells and have yet to be shown to establish persistent infections. Meinkoth and Kennedy (1980), using SFV, found that persistent infections could either

be established with an inoculum containing a high concentration of DI particles, or pretreatment of cells with interferon prior to inoculation of S virus. Similarly Weiss et al. (1980) established a persistent infection using DI SV. Once established, persistently infected cells released temperature sensitive virus. The DI particles used to establish the persistent infection failed to interfere with the temperature sensitive virus, but would interfere with the multiplication of SFV (Weiss and Schlesinger, 1981). Barrett and Atkins (1981) have used mutant S virus (SV) to establish a persistent infection. It would appear that persistent infections can involve either one or more of the mechanisms listed above.

In contrast to the acute virus infections in vertebrate cells, alphaviruses readily establish persistence in mosquito cells (for a review see Stollar, 1980). High levels of virus are produced with little or no cpe. Temperature sensitive mutants (Shenk and Stollar, 1974) and DI particles (Igarashi et al., 1977; Eaton, 1977; Tooker and Kennedy, 1981) have been implicated as causal agents.

h) Animal Studies

i) History

Although there is a vast literature on the biochemistry of DI particles, few in vivo studies have been reported. Some of the earliest work on influenza DI particles by von Magnus described the role of "incomplete" virus of the A/PR8(HINI) strain in ovo and in mice (von Magnus, 1951b).

Intranasal inoculation of dilutions of "incomplete" virus of this neurotropic strain resulted in mice dying from paralysis, while using the same route of inoculation, undiluted "incomplete" virus gave no paralysis and the mice survived. These experiments were probably the first to show an effect of DI viruses in virus infections of animals.

The possibility that DI virus may play a role in the expression of virus diseases was suggested by Huang and Baltimore in 1970. They proposed that DI particles could be important in determining the course of both acute and persistent virus infections in nature and speculated on their possible use as antiviral or prophylactic agents.

Modulation of infections of animals by DI particles has been described for a number of virus groups and these are described below.

ii) Rabies virus

Rabies virus, produced by undiluted passage in eggs, was found to be apathogenic after intracerebral inoculation in young adult mice, while dilutions of this virus killed mice (Koprowski, 1954). These experiments have been confirmed recently by studies upon similar rabies virus preparations (Wiktor et al., 1977). However, since the latter studies did not have proper controls for the immunogenic effects of DI virus, it is uncertain if protection was the result of intrinsic interference. Other work has questioned the relationship between DI virus and virulence since Wunner and Clark (1980) found no correlation between virulence of different strains of rabies virus and their ability

to generate DI particles.

iii) Rift Valley Fever virus

Mims (1956), working with the bunyavirus Rift Valley Fever virus, observed that inoculation of "incomplete virus" lengthened the incubation period of the disease and reduced infectivity titres in mice. The "incomplete" virus was also shown to be capable of immunising mice since high titres of neutralizing antibody were obtained after its administration. Mice were also protected by intracerebral inoculation of S virus and intravenous inoculation of "incomplete" virus.

iv) Vesicular stomatitis virus

a) Intracerebral inoculation

Holland and co-workers have published a series of papers describing the modulation of disease caused by VSV in mice by DI particles. The first experiments, described by Doyle and Holland (1973), showed that purified DI VFV can protect young adult mice against the lethal encephalitis caused by intracerebral inoculation of S virus. However, large quantities ($>10^{10}$) of DI particles were required and when inoculated by the intracerebral route these protected against small, but nonetheless fatal, doses of S virus. Co-inoculation with higher doses of S virus only resulted in an increase in survival time before death ensued. Holland and Doyle (1973) also showed that DI virus reduced S virus multiplication in the brain but failed to directly detect DI particles in the brains from adult mice. These studies attributed protection to

DI particles since protection was specific for homologous (Indiana serotype) virus and not heterologous (New Jersey serotype) virus, and no induction of interferon could be demonstrated. Further studies by Holland and Villarreal (1975) showed that newborn mice were also protected from VSV infection after intracerebral inoculation by DI VSV, but only low titres of DI VSV were present in protected mice. Possibly this is due to the low levels of virus multiplication in the brains of protected mice (Doyle and Holland, 1973). Generation of DI VSV could be demonstrated in the brains of adult mice after 2 serial undiluted passages in brain, however DI virus was only detected by pooling mouse brains and using *in vitro* amplification assays. From these results it was concluded that DI particles protected mice by their ability to interfere with the multiplication of S virus. No persistence of infectious virus was observed in protected mice, however some mice developed a slow progressive disease with death being greatly delayed.

Rabinowitz *et al*. (1977) confirmed the studies of Holland and colleagues using the same strain of mouse as Holland *et al*. but a different isolate of DI virus. They observed a slow progressive disease of the central nervous system after intracerebral co-inoculation of DI and S VSV. Histopathological examination of the brains from infected mice showed many pathological changes not associated with S virus infection. These workers used 10^{11} DI particles to achieve modulation of the S virus infection (1.5×10^{5} pfu) and this is typical of the studies with DI VSV where enormous numbers of DI particles are required to achieve protection. Holland *et al*. (1978) quote a minimum number of "3×10^{9} physical particles of active DI virus" for protection.

In 1977, Crick and Brown repeated the experiments of Holland and associates and demonstrated that mice were protected by administration of acetyl ethyleneamine-inactivated DI VSV. Also DI VSV protected against heterologous strains of VSV, rabies and a neurotropic strain of foot-and-mouth disease virus. Crick and Brown proposed that the protection observed was due to activation of the host defence responses and not intrinsic interference by DI virus. Although both sets of workers used the Indiana strain of VSV, it is possible that the different results are due to the use of different types of DI particles and mice strains by the two groups. Since generation of DI RNA is a random process (Holland et al., 1976) it is conceivable that one DI RNA may function in the animal, while another may not. Although Holland and co-workers did not study host immune responses or include all the controls that their experiments demanded, a recent study by Jones and Holland (1980) has demonstrated that biologically active DI particles, and not UV inactivated DI or S virus, protect mice against challenge by VSV S virus. Hence protection would not appear to be due to a host immune response. Some in vitro studies on DI VSV by Faulkner et al. (1979) also support the results of Holland and co-workers. They reported that co-infection of cultured neurones from mice delays death and suppresses virus growth. These observations suggest that DI particles may play a role in virus infections by interfering with the multiplication of S virus.

b) Intraperitoneal inoculation

In comparison to the above studies which have looked at infections of the central nervous system, Fultz et al. (1982a) looked at the systemic

infection of Syrian hamsters by VSV after intraperitoneal inoculation. They reported that biologically active DI VSV can protect hamsters against the lethal S virus infection. As with previous studies with DI VSV, large amounts ($\geq 10^7$) of DI particles were required to achieve significant protection. Protection resulted in low levels of S virus in serum and tissues. Fultz et al. (1982a) suggest that protection is not only mediated by the intrinsic interfering capacity of DI virus. Since they demonstrated heterologous interference against the New Jersey serotype of VSV and induction of interferon by DI VSV in mice. Protection was also observed when DI and S virus were inoculated by different routes. In another study, Fultz et al. (1982b) established persistent infections in Syrian hamsters after intraperitoneal inoculation of DI and S virus. Virus was detected by co-cultivation techniques upto 8½ months after inoculation, but preliminary studies indicated that the viruses isolated were temperature sensitive, small plaque mutants.

v) Semliki Forest virus

These are the only in vivo experiments with DI alphaviruses. Dimmock and Kennedy (1978) observed that co-inoculation of DI and S SFV by the intranasal route resulted in modulation of the lethal encephalitis caused by virulent S SFV. Mice either survived infection without showing any clinical signs of S virus infection ("protection") or died following the normal SFV pattern of disease. Simultaneous inoculation of the DI virus and the S virus was necessary for protection. DI SFV reduced multiplication of S virus in the brain by at least 10^5 fold and

DI virus was detected in the brain after amplification in tissue culture (which are both similar to that observed in the VSV system). In comparison to the VSV system where large numbers of DI particles are required for protection, 2×10^5 "pfu equivalents" of biologically active DI SFV particles were required to protect the majority of mice against 10 LD_{50} (6000 pfu) S virus. Dimmock and Kennedy (1978) also showed that protection was not due to the induction of interferon or stimulation of the host immune system. They concluded that protection of mice ws due to the interfering capacity of DI SFV. Recent studies by Crouch et al. (1982) have shown that the brains of protected mice have no pathological or histochemical lesions whatsoever, nor was there any evidence of mononuclear cell infiltration. These results would seem to support the view of Dimmock and Kennedy (1978) that DI SFV protects mice by intrinsic interference rather than by the host's immune processes. However, something other than intrinsic interference eventually eliminates S virus from the brain.

vi) Influenza virus

After the original work of von Magnus (1951b) [see section (i)], the next study on influenza DI virus in animals was by Holland and Doyle (1973). These workers showed that co-inoculation of DI and S virus of the A/NWS strain of influenza (H1N1) by the intranasal or intracerebral routes of inoculation did not prevent death but delayed the onset of clinical signs of infection and reduced infectivity titres in the lungs. Gamboa et al. (1976) inoculating the A/WSN strain (H1N1) by the intracerebral route found that mice could be protected by DI virus.

They confirmed the studies above and also showed that modulation was dependent upon the age of the mice. Seven week old mice were protected while the virus titres were only reduced in 3 week old mice. Unfortunately the studies did not include controls for the immunogenic load of the inoculum and protection may have resulted from stimulation of the immune response. Frolov et al. (1978) showed that intranasal inoculation of mice with DI virus of the A/Hong Kong/1.68 strain (H3N2) reduced the acute respiratory disease caused by S virus. Similar results were obtained by Kantorovich-Prokudina et al. (1979) for the A/WSN strain of influenza virus. Rabinowitz and Huprikar (1979) showed that pulmonary infection of mice by the intranasal inoculation of A/PR/8 strain of influenza virus was prevented by administration of DI virus. However, it was mouse strain specific and was correlated with a humoral immune response rather than interference with S virus replication.

vii) Arenaviruses

These studies are particularly interesting as arenaviruses naturally persist in their rodent hosts. Popescu and Lehmann-Grube (1977) observed that DI virus was present in both acute and persistent infections of mice with LCMV. Welsh et al. (1977) have shown that intracerebral administration of DI LCMV prevented the central nervous system disease caused by S virus in 2 day old rats. The synthesis of S virus and LCMV antigens was reduced while no interferon or host immunological responses appear to have been involved in modulation. Aggregation of DI virus enhanced protection and no persistent virus was observed at 6 weeks p.i. Since the disease caused by S virus is immune

mediated these workers propose that the modulation correlates with the ability of DI LCMV to block synthesis of surface antigens in infected cells (Welsh and Oldstone, 1977). Coto (1980) has shown that DI virus is generated in newborn mice infected with Junin virus and proposes that this is responsible for the observed delay in death and the reduced yield of S virus in co-infected animals.

viii) Reovirus

Spandidos and Graham (1976) demonstrated the generation, replication and in vivo interfering ability of DI reovirus in newborn rats after intracerebral and subcutaneous inoculation. They claimed that DI virus was generated during the acute phase of disease and such DI virus was generated from the L_1 segment only. Surviving rats were runted and chronically infected, and DI virus present in these brains contained virus RNA segments with multiple deletions. All but two of the chronically infected rats also contained virus which was identical to that in the inoculum. The claim that DI virus was generated de novo is difficult to substantiate as it cannot be proved that the inoculum ws free of DI virus. Thus the question of in vivo generation of DI virus in this system remains unanswered.

ix) Runde virus

Traavik (1978) has reported persistent infections of mice after intracerebral inoculation of the coronavirus Runde virus into suckling mice. He has suggested that this persistence is due to DI virus.

x) Flaviviruses

Darnell and Koprowski (1974) proposed that DI particles may be involved in the genetic resistance of mice to infections by West Nile virus initiated by the intraperitoneal route. Cells from resistant mice produced interfering particles whereas cells from susceptible mice did not. Brinton (1983) has confirmed these studies and shown that cell cultures from resistant mice produce and amplify DI particles more efficiently than cell cultures from susceptible mice. Smith (1981) has implicated "interfering virus" as being involved in the genetic resistance of mice to infection by Banzi virus. After intraperitoneal inoculation, mice resistant to infection were found to have high levels of "interfering virus", while susceptible strains of mice had much lower titres. Surprisingly, "interfering virus" was detected in the spleen and not the brain, and cyclophosphamide enhanced the "interfering virus". From these results Smith has proposed that "interfering virus" originates in cells of the lymphoreticular system.

xi) Significance of animal studies

From the above it is clear that in many different systems DI viruses can alter the normal expression of disease resulting in modulation of infections either with complete protection of the animal or a delay in death. The role of DI viruses in virus infections of animals appears to be complex since the studies described above indicate that the age and strain of the animal, route of inoculation, amount of S virus and quantity and properties of DI virus can all affect the interaction between DI and S viruses in vivo. Interpretation of some studies is

difficult due to the immunogenic load of the DI virus used in the inoculum, but other studies have shown that this need not be a problem if proper controls are used. It is unfortunate that many studies have used intracerebral inoculation as this can infect the blood stream simultaneously, and has several other drawbacks (Mims, 1964).

The quantities of DI virus inoculated into animals to achieve protection against S virus tend to be large. For example, Jones and Holland (1980) quote that approximately 10^8 DI particles were required to protect against a lethal infection caused by intracerebral inoculation of 100 pfu. This is to be expected as DI virus is not self-replicating, and is required to enter the same cell as S virus to exert interference. What is surprising is that with so many cells available for infection that co-infection of cells with DI and S virus should occur at all.

The DI virus-modulated infection may be more complicated than appears at first sight, as it seems that DI viruses are not the only variants which can alter the pattern of disease: in particular temperature sensitive mutants also have this property *in vivo* (Haspel *et al.*, 1975; Clark and Ohtani, 1976; Stanners and Goldberg, 1975). The possibility that DI viruses themselves are also temperature sensitive must also be considered. Serial undiluted passage which is used to propagate DI virus also results in the accumulation of various S virus mutants including temperature sensitive mutants, and Holland *et al.* (1982) have reported the evolution of both DI and S virus during persistent infections in tissue culture. These points must all be borne in mind in any discussion of modulation of virus infections.

xii) DI viruses and natural infections

It is clear that DI viruses can be generated and enriched in tissue culture, but to date there has been no report of the clinical isolation of DI virus from any natural virus infection, either of man or any other animal. Until such time as DI virus is clinically isolated, its role in the modulation of virus infections must be treated with caution. It should be emphasised also that, although a number of *in vivo* model systems have been described for the role of DI particles in virus infections, they all involve viruses which have either been adapted for growth in tissue culture or have been passaged in animals prior to use in experiments. Thus, the relevance of studies of these laboratory viruses to natural infections caused by viruses must be questioned. This should not, however, distract from the value of model systems in helping to understand the potential of DI particles for modulating virus infections *in vivo*.

Is there any evidence that DI viruses may actually play a role in modulating a virus infection? One consideration must be the persistent infections caused by hepatitis B virus in which a role for DI virus has been suggested (but without any evidence) by Robinson (1978). More recently, Ruiz-Upano *et al*. (1982) have looked at the structure of hepatitis B virus DNA and suggested that supercoiled DNA is present in infectious virus while relaxed circular form DNA in Dane particles may represent DI particles. It has also been postulated that persistence of arenaviruses in small rodents may be caused by DI particles, but no evidence exists to support this theory (see review by Buchmeier *et al*., 1980). From time to time, it is suggested that measles virus infection

is modulated to subacute sclerosing panencephalitis by DI particle production, however there is no evidence to support this speculation (Hall et al., 1974; Cernesa and Sordi, 1980).

Finally, two isolates of human rotavirus from immunodeficient children have been found to have unusual genomes. Separation of the RNA segments by polyacrylamide gel electrophoresis has shown the presence of 3 additional RNA species to the 11 segments observed in rotavirus virions. The extra segments were found not to be due to mixed rotavirus infections and cloned recombinant DNA rotavirus probes hybridized not only to the normal virion genomic RNAs but also to RNA species of both higher and lower molecular weights (S. Pedley, personal communication). Tissue culture studies with calf rotavirus have shown that serial undiluted passage results in the isolation of virus particles with extra RNA segments is additional to the normal virion RNA species. Like the two isolates described above, the extra RNA species had both larger and smaller molecular weights than the virion genomic RNA species (S. Pedley, personal communication). Thus it is tempting to suggest that the two clinical isolates may be DI particles. Unfortunately, these clinical isolates cannot be shown to have interfering activity as they cannot be propagated in tissue culture.

Materials and Methods

Source of Chemicals

L-[^{35}S]methionine (specific activity 800 Ci/mmol), [^{32}P]orthophosphate (80-130 Ci/mg phosphorus), [methyl-^{3}H] thymidine (25 Ci/mmol) and [5'-^{3}H]uridine (30 Ci/mmol) were obtained from Amersham International plc, Amersham, Bucks.

Acrylamide and N-N'-methylene bisacrylamide (specially pure grade) were obtained form BDH Chemicals Ltd., Poole, Dorset, U.K. N,N,N',N'-tetramethylenediamine (TEMED) was purchased from BioRad Laboratories, Richmond, California, USA. 2,5-diphenyloxasole (PPO), 2,2'-p-phenylene-bis]5-phenyloxasole] (POPOP) and toluene were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.

Fuji Rx X-ray film and Kodak X-Omat S were obtained from Fuji Photo Film Co. Ltd., Tokyo, Japan and Kodak Ltd., Liverpool, U.K. respectively.

Actinomycin D was obtained from Merck, Sharpe and Dohme, Rahway, New Jersey, USA. DEAE (Diethylaminoethyl)-dextran and agarose (Type II, high EEO) were purchased from Sigma Chemical Co., St. Louis, USA. Noble agar was obtained from Difco Laboratories, East Molesey, Surrey., Soluene-350 tissue solubiliser was obtained from Packard Instrument Co. Inc., Illinois, USA.

Flat-bottomed glass vials (50 x 12mm) were obtained from Regina Industries Ltd., Stoke-on-Trent, U.K. All media and newborn calf serum were purchased from Flow Laboratories. Irvine, Scotland.

Cells

HeLa, BS-C-1, human foreskin fibroblasts (HFF) and L_{929} cells were cultured in Glasgow's modification of Eagles medium (Eagle, 1959), supplemented with non-essential amino acids (GMEM-NEAA) containing 10% (v/v) NCS. BHK-21 cells were grown in GMEM supplemented with tryptose phosphate broth (GMEM-BHK) containing 10% (v/v) NCS. Cultures of primary chick embryo fibroblast (CEF) cells were prepared as described by Morser et al. (1973). Cultures were maintained in medium 199 plus 5% (v/v) NCS.

Cells were cultured in monolayers on either glass or tissue culture plastic (Nunclon (Gibco, U.K.)) flasks. All cells were grown in an atmosphere of 5% CO_2/95% air at 37°C. All media contained antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml).

Confluent monolayer cultures were prepared for subculturing by removing the culture medium and washing the cells with warm phosphate buffered saline (PBS, pH 7.4). Cells were detached from the substrate by incubating with 5 ml 0.05% (w/v) Trypsin (Flow Laboratories, U.K.), 0.02% (w/v) versene (1:5 v/v) mixture at 37°C until the cells had detached. Cells were gently dispersed by pipetting in 10 ml medium, counted in a Neubauer haemocytometer, and seeded at the required concentration into fresh flasks containing pre-warmed growth medium.

All media, trypsin and versene were sterilised by membrane filtration through three 0.22 µm Millipore filters (Millipore UK Ltd., London).

Storage of frozen cells

Cells were frozen in medium containing 20% (v/v) NCS and 10% (v/v) dimethyl sulphoxide. Ampoules were wrapped in cotton wool to ensure the temperature decreased slowly and placed at -70°C overnight and then placed in liquid nitrogen.

Virus

A plaque purified stock of the virulent ts^+ strain of SFV was originally obtained from Professor F. Fenner (John Curtin School of Medical Research, Australian National University, Canberra), and was first reported by Tan et al. (1969). This was grown in mouse brain by intracerebral inoculation and plaque purified three times. A stock was then prepared in a subconfluent roller bottle culture of BHK-21 cells and virus was harvested after 48 h at 33°C. This virus stock was designated passage zero (p0). Unless otherwise noted, this is the strain of SFV used to derive the DI and standard virus stocks used in the thesis.

Virulent L10 strain (Bradish et al., 1971) and avirulent A774 strain (Bradish et al., 1971) of SFV were obtained from Dr. R. Fitzgeorge and Dr. C. Bradish (Porton Down., Wilts.). Sindbis (originally from Dr. J. S. Porterfield, NIMR, Mill Hill, London) and encephomyocarditis (EMC) (from Dr. E. Martin, NIMR, Mill Hill, London) viruses were obtained from laboratory stocks. EMC virus was grown in HeLa cells (moi of 0.1) for 18 h at 37°C, and the other viruses in BHK cells (moi of 0.1) for 18 h at 37°C.

Standard virus stocks

p0 Virus of the ts^+ strain of SFV was used as seed for making p1 or standard (S) virus stocks. Roller bottle cultures of BHK-21 cells were inoculated with a moi of 0.1 of p0 virus. After 1 h at 33°C, the inoculum was removed and 100 ml of maintenance medium (GMEM-BHK + 2% (v/v) NCS) was placed in the roller bottle. Extracellular virus was harvested after 18 h at 33°C. Tissue culture fluids were clarified by centrifugation at 2000 rpm for 15 min at 4°C. Virus was then aliquoted and stored at -70°C.

DI virus preparations

DI virus preparations of ts^+ strain of SFV were all derived by serial undiluted passage, usually in BHK cells, with the addition of enough S virus to maintain constant moi of 50 pfu/cell. Cultures were incubated for 24 h at 37°C and extracellular virus harvested. DI virus preparations are all designated by the number of undiluted passages and a small letter to indicate sister stocks (e.g. p13d, p13h). Where appropriate the passage history of each preparation is shown by the number of passages in BHK (B), primary chick embryo fibroblast (C) or L_{929} (L) cells. All DI virus prparations which had in excess of.8 undiluted passages (DI virus p9, p10, p11, et.) were derived from DI virus p8 used by Dimmock and Kennedy (1978). DI virus preparations below p8 were generated independently from S virus as described above.

DI virus of L10 and A774 strains of SFV and SV were obtained by inoculating BHK cells with S virus at a moi of 50 and harvesting after

48 h at 37°C. This preparation, p1, contained DI virus as previously demonstrated for the ts^+ strain of SFV (Barrett et al., 1981). DI virus p1 was propagated together with a moi of 50 of S virus for 24 h at 37°C in BHK cells and designated p2.

Plaque assay of SFV

SFV was assayed by plaque formation on confluent monolayers of primary CEF cells in 5 cm plastic Petri dishes. Virus was diluted in medium 199 plus 2% (v/v) NCS and 200 µl samples were inoculated onto plates. After 1 h at 33°C, inocula were removed and the cells overlaid with medium 199 containing 0.9% (w/v) noble agar, 2% (v/v) NCS and 0.04% (w/v) DEAE-dextran. Plaques were visualised after 2 days incubation at 33°C by ovelaying the agar with 3 ml neutral red strain (0.01% (w/v) in PBS) for 2 h at 33°C.

Interference assays

i) RNA synthesis inhibition assay

Mouse L_{929} cells (2×10^5) were seeded in flat-bottomed glass tubes and were used after overnight incubation at 37°C. Assay dilutions were done in quadruplicate. DI SFV was diluted into medium containing S virus (moi of 50) and 2 µg/ml actinomcyin D. Control tubes were inoculated with S virus only or mock infected. After inoculation (250 µl/tube) for 1 h at 37°C, medium was removed and monolayers washed once with warm PBS. Medium (GMEM, 250 µl) containing 2% NCS and 2 µg/ml actinomycin D

(maintenance medium) was placed in each tube. At 4 h p.i., the maintenance medium was removed, monolayers washed once with warm PBS and replaced with 250 µl maintenance medium containing 10 µCi/ml [^{3}H]-uridine. After 1 h at 37°C, the radioactive medium was removed and the tubes were then processed to determine incorporation of TCA insoluble [^{3}H]-uridine into virus RNA as described by Barrett et al., (1981).

ii) Yield reduction assay is described in Figure 7.

UV irradiation

This was performed according to Dimmock and Kennedy (1978) except that the virus was placed 10 cm below a UV lamp (Gelman Sciences Ltd., Northampton, U.K.). The dosage was 14 µw/sec/cm^2 except for the UV irradiation curve for DI virus p13 where it was 8 µw/sec/cm^2. The dosage was checked each time using a Blak-Ray UV meter (Model J225, UV Product Inc., San Gabriel, California, USA).

Inteferon Assay

Interferon was assayed by the viral RNA inhibition technique of Atkins et al. (1974). This method was modified by using flat-bottomed glass tubes. Interferon samples were diluted from 10^{-1} to 10^{-5} in steps of $10^{-0.5}$. Challenge virus was inoculated in 250 µl of maintenance medium and virus RNA synthesis was detected by adding 2.5 µCi [^{3}H]-uridine to each sample. Three ml of acidified scintillant was added to each sample

prior to counting in a Packard liquid scintillation spectrometer. All assays included an internal interferon control of known titre.

To remove biologically active virus from interferon samples, samples were dialysed for 5 days at pH2 (152 mM HCl, 87 mM KCl) at 4°C. Samples were then returned to pH7 by dialysing overnight against PBS at 4°C.

Haemagglutination assay

This was performed according to Clarke and Casals (1956). A pH of 5.9 was critical and was maintained by use of phosphate buffer. Goose cells were obtained from CAMR, Porton Down, Salisbury, Wilts.

Radiolabelling of intracellular virus polypeptides

Monolayers of L_{929} cells in flat bottomed tubes (prepared as for the YRA (Figure 7) or Petri dishes, were rinsed with PBS and incubated overnight at 33°C in GMEM containing 2% NCS and 2 µCi [^{3}H]-thymidine to radiolabel cell DNA. Medium was then removed and the monolayers rinsed with warm PBS. Samples were prepared and diluted in methionine-free GMEM medium as necessary. Monolayers were inoculated with samples and incubated for 1 h at 37°C. The inocula were removed and replaced with methionine-free GMEM medium and incubated at 37°C until 4 h p.i. Medium was then removed and replaced with 50 µCi [^{35}S]-methionine in 100 µl methionine-free GMEM medium. After 1 h at 37°C, the medium was removed and the cells rinsed with PBS, frozen in dry ice/methanol and thawed and dissolved in detergent (10% SDS, 10 mM Tris-HCl, pH7.4). The amount of

^{3}H-thymidine incorporated was then determined so that the number of cells used for analyses of virus polypeptides could be standardised.

Analysis of virus polypeptides by polyacrylamide gel electrophoresis (PAGE)

Polypeptides were resolved on a 3 to 25% gradient of polyacrylamide gel with a reversed linear bis-acrylamide gradient of 0.4 to 0.12%, using the buffer system of Laemmli (1970). This system was used when it was discovered that the 10 to 30% linear gradient gel system of Cook et al. (1979) failed to resolve all the SFV polypeptides. The gel in Figure 13a was a 10 to 30% linear gradient according to Cook et al. (1979). Electrophoresis was at 80V for about 16 h. After washing, gels were dried under vacuum and exposed for autoradiography. Gel bands were cut out and radioactivity determined after incubating gel slices in 3 ml toluene scintillation cocktail (9% NCS tissue solubiliser (Amersham-Searle), 0.4% (w/v) PPO, 0.005% (w/v) POPOP, 1% water in toluene) at 37^{o}C for 48 h and then overnight at 4^{o}C.

Measurement of virus RNA synthesis

Identical conditions to the RNA synthesis inhibition assay were used except that cultures were radiolabelled for 1 h periods at various times p.i. Incubation was stopped by snap-freezing monolayers in a dry ice/methanol bath.

Preparation of virus particles with radio-labelled RNA

[^{3}H]-uridine labelled virus RNA was prepared by propagating virus as described above, but with the addition of [^{3}H]-uridine and actinomycin D (2 µg/ml) to the culture medium. [^{32}P]-orthophosphate labelled virus was prepared by starving cells of phosphate in phosphate-deficient medium supplemented with 2% dialysed NCS. Culture were infected and incubated in phosphate deficient medium containing 10 mCi [^{32}P]-orthophosphate. Virus was grown in the presence of actinomycin D (2 µg/ml). After incubation the tissue culture fluid was clarified by centrifuging in a MSE Chilspin at 3000 g for 15 min at 4°C and then virus pelleted in a ultracentrifuge. RNA was extracted from the virus as described by Clewley et al. (1982).

Gel electrophoresis of virus RNA

RNA species were resolved by electrophoresis in 1% (w/v) agarose gels in 50% (v/v) formamide by using a horizontal, submerged gel system (Clewley and Avery, 1982). Prior to electrophoresis the RNA sample was dissolved in water and an equal volume of formamide added. Samples were heated at 65°C for 1 min immediately before loading on the gel. After electrophoresis for 8 h at 120V, the gel was fixed in 1 M acetic acid. Water was removed from the gel by three sequential 1 h washes in methanol. The gel was then soaked for 3 h in 3% (w/v) PPO in methanol, for 1 h in water to precipitate the PPO and then dried under vacuum. Kodak X-Omat S film was pre-exposed for fluorography as described by Laskey and Mills (1975) and exposed to the gel at -70°C.

Oligonucleotide mapping of RNA

The digestion of [^{32}P]-labelled RNA by T_1 ribonuclease and the resolution of oligonucleotides by 2-dimensional PAGE were carried out by the procedures of Clewley et al. (1977).

Mice

CFLP and Porton random bred white mice 4 to 5 weeks old, weighing about 25 g, were obtained from Hacking and Churchill Ltd., Huntingdon, Cambs., and Allington Farm, Porton Down, Salisbury, Wilts. respectively and were used 3 to 6 days after delivery. C_3H-He/Mg inbred mice came from the Warwick University Breeding colony and weighed about 18 g at 5 weeks of age. All mice used were male except where stated.

Inoculation of Mice

Intranasal (i.n.). All mice were inoculated under light ether anaesthesia with a 20 µl volume as described by Dimmock and Kennedy (1978). Treatment of mice with DI SFV comprised 2 inoculations of a DI SFV preparation 2 h apart. The second inoculation contained 10. LD_{50} S SFV. Control mice were inoculated in exactly the same way with non-infectious UV-irradiated S SFV replacing DI virus, in order to control for possible immunogenic effects and to demonstrate that the mere presence of SFV antigen did not prevent infection (see Results). Other controls received DI virus alone, UV SFV alone or diluent.

Intraperitoneal (i.p.). Mice were inoculated intraperitoneally with a 100 µl volume of DI virus containing 10 LD_{50} S virus (1.25×10^5 pfu/LD_{50}). Control mice were inoculated with 10 LD_{50} but with non-infectious UV-irradiated S SFV replacing DI virus, in order to control for possible immunogenic effects.

Tissue sampling

When tissue samples were required mice were killed with ether. Blood was obtained from the heart and the serum was then removed and stored at -70°C. Spleen, olfactory lobes and brain (minus olfactory lobes) were dissected out and dispersed in medium 199 containing 2% NCS by passing tissue through a 19 gauge syringe needle and the supernate was stored at -70°C. Olfactory lobes and brain were pooled for tissue sampling following i.p. inoculation.

Myocrisin treatment

Myocrisin (sodium auro-thio-malate, May and Baker, Dagenham, U.K.) was administered i.p. at a dose of 400 mg/Kg body weight in a volume of 200 µl physiological saline 3 h before inoculation of virus. This dose was the maximum that could be administered without killing the mice.

Neutralizing antibody assay

A plaque reduction assay was used. Brain homogenate or serum was diluted in GMEM plus 2% NCS containing 50 pfu of virus and incubated for

1 h at 25°C. The mixtures were then plaque assayed. The titre of neutralizing antibody was taken as the reciprocal of the dilution which gave 50% reduction in plaques when compared to a control containing 50 pfu and medium only.

Results

Chapter 1

Heterogeneity of interference properties displayed by DI SFV in cell culture

Introduction

To date five types of biological assay for measuring DI virus have been described: a) reduction in yield of standard virus (first described for VSV, Bellett and Cooper (1959)); b) inhibition of infectious centre formation (first reported by Welsh et al. (1972) for lymphocytic choriomeningitis virus); c) a focus-forming assay which measures the ability of DI virus to allow individual cells to survive and proliferate (first described for DI lymphocytic choriomeningitis virus, Popesiu et al., 1976); d) a colorimetric assay based on neutral red dye uptake by DI particle protected cells described by Treuhaft (1983) for respiratory syncytial virus. All these assays measure the inhibition of the production of standard virus progeny. The fifth assay e) is based on the ability of DI SFV to inhibit synthesis of virus-specified RNAs (Barrett, 1980; Barrett et al., 1981). The latter assay, known as the RNA synthesis inhibition assay (RSIA), is based on the observation that co-infection of cells with DI and S virus results in inhibition of a virus RNA synthesis (Guid and Stollar, 1975; Barrett et al., 1981). The RSIA differs from the other four assays described and it was decided to compare it with a yield reduction assay to see if the assays were measuring the same aspect of interference.

Results

a) Yield Reduction assay

The yield reduction assay (YRA) for DI SFV measures interference with the yield of infectious progeny virus and follows the design of Bellett

and Cooper (1959) and Kowal and Stollar (1980). Cells were grown in flat-bottomed glass tubes (50 x 12 mm, Regina Industries Ltd., Stoke on Trent, U.K.) for the assay which is described in detail in Figure 7. This is the "optimized interference assay". In this assay and all other experiments reported below, actinomycin D was used to prevent the possibility of induction of interferon.

Figure 8 shows that the decrease in progeny virus was proportional to the concentration of DI virus. The interference titre of DI SFV is defined as the reciprocal of the dilution which causes a 50% reduction in the yield of infectious progeny compared with cultures infected with S virus alone, and is expressed in "defective interfering units" or DIU. Thus DI SFV titrated in Figure 8 has a titre of $10^{1.6}$ DIU/250 µl (the volume of the assay) or $10^{2.2}$ DIU/ml.

Assuming that at least one DI particle (DIP) per cell is necessary to completely inhibit S virus synthesis by that cell, the minimum number of DIPs detectable by the assay can be found from the Poisson distribution (see below) since infection of a cell by a DIP is a random event.

$$P(r) = e^{-m} \cdot \frac{m^r}{r!}$$

Where P(r) = the fraction of cells in the flat-bottomed tube receiving r DIPs where the average ratio of DIP:cell, i.e. the moi, is m.

Therefore, when there is 50% reduction in S virus synthesis P(r) = 0.5 and P(r) that cells are not infected by a DIP is r = 0.

Figure 7. The Optimized yield reduction assay procedure

1. Tubes are soaked in distilled water overnight, dried, placed in trays and sterilized in dry heat at 160°C for 4 h.

2. Tubes are seeded with 2 x 10^5 cells per vial in 1 ml GMEM + 10% NCS (growth medium) and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. This gives confluent monolayers.

3. The following day the medium is removed and the cells washed x 1 with PBS. Tubes are then aspirated x2.

4. $10^{0.5}$-fold dilutions of DI virus sample from 10^0 to 10^{-4} are made in a diluent of GMEM + 2% NCS + 2 µg/ml actinomycin D (maintenance medium) containing S virus at a moi of 5. Virus controls are maintenance medium containing S virus at a moi of 5.

5. 250 µl aliquots of each dilution and control are placed in vials in quadruplicate.

6. After 1 h at 37°C the inoculum is removed and replaced with 1 ml maintenance medium.

7. At 16 h p.i. the culture fluids are harvested and infectivity measured by plaque assay.

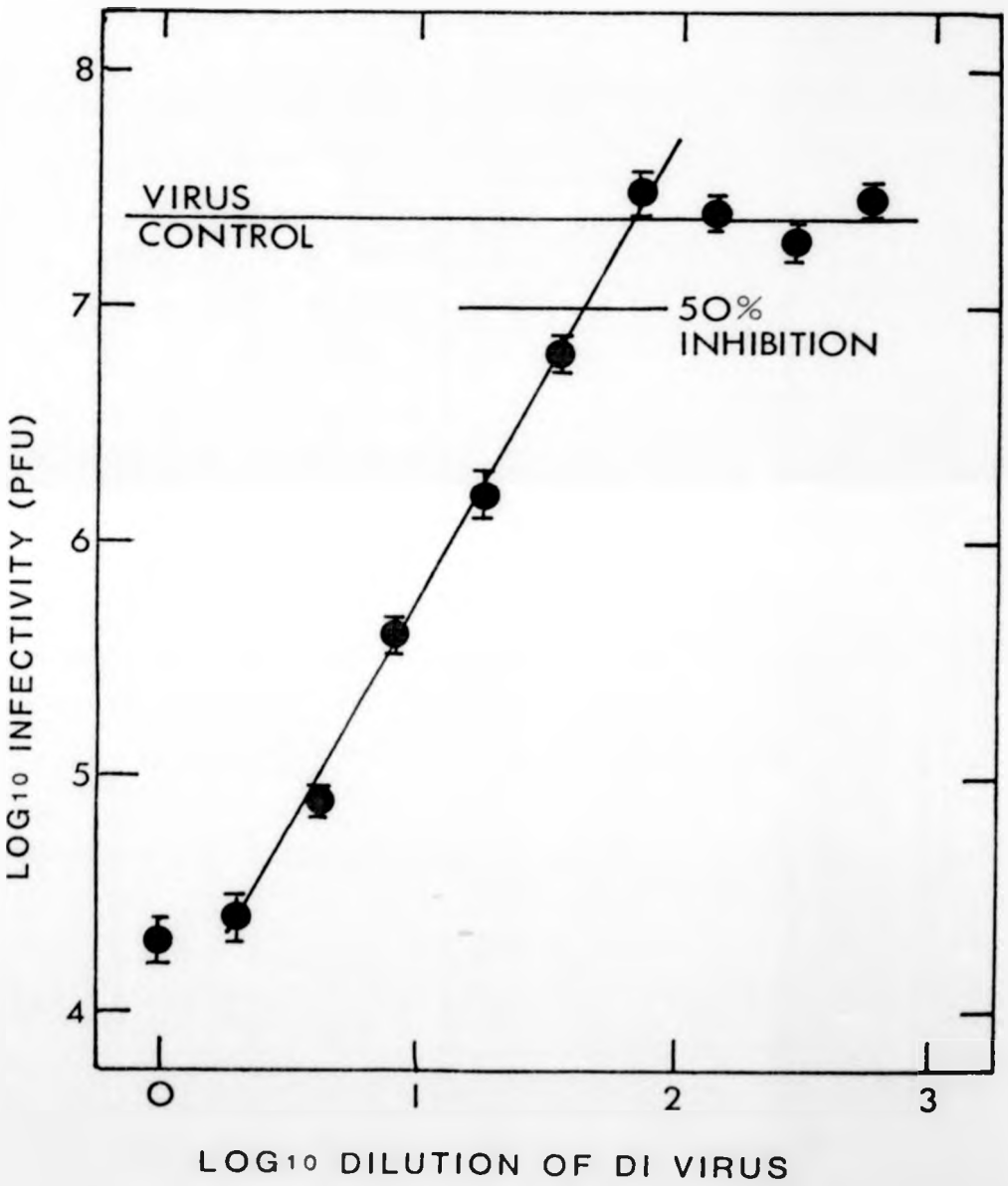

VIRUS
CONTROL
50%
INHIBITION
8
7
6
5
4
0
1
2
3
LOG10 INFECTIVITY (PFU)
LOG10 DILUTION OF DI VIRUS

Figure 8. A yield reduction assay (YRA) measuring the decrease in yield of infectious progeny brought about by DI SFV

This assay is described in Figure 7. Virus controls represent cultures infected with S virus only. Error bars show ± 1 standard error of mean.

Therefore, $Pr = 0.5 = e^{-m} \cdot \frac{m^0}{0!}$

$m = 0.7$

Therefore on average 70% of the cells must be infected by one DIP. The minimum number of DIPs detectable is 70% of the number of cells in a flat-bottomed tube = $0.7 \times 2 \times 10^5$. Therefore the assay will detect a minimum of 1.4×10^5 DIPs. Hence the interference titre calculated from Figure 8 is $10^{2.2}$ DIU/ml and this represents a minimum of $10^{7.4}$ DIP/ml.

b) Optimisation of the YRA

i) Cell Type

It is known that interference by DI viruses is expressed to different extents in different cell types (Stark and Kennedy, 1978; Barrett et al., 1981). Hence this was investigated in order to obtain the most sensitive cell system for the YRA (Table 3). Cell types could be ordered to display a gradient of interference by the DI virus with L_{929} giving the highest interference titre and no detectable interference in chick embryo fibroblasts. A similar spectrum was obtained with the RSIA (Barrett et al., 1981). It is interesting that DI SFV can be propagated in CEF cells even though it exerts no interference in these cells as measured by either assay.

ii) Temperature, incubation time and m.o.i.

Maximum interference titres were attained by using a moi of S virus of 5

Table 3 Interference titres of DI SFV p11 by the YRA in different cell types*

Cell Type	Origin	Interference Titre (DIU/ml)
L_{929}	mouse	140
BHK	hamster	63
BS-C-1	monkey	20
HFF	human	20
CEF	chicken	≤ 4

* Assays were carried out as described in Methods except that different cell types were used. All vials contained 2×10^5 cells except for CEF cells where 6×10^5 cells were used.

Table 4 Optimisation of the YRA

Temperature of incubation	Time of harvest (h pi)	moi of S virus	Interference titre (DIU/ml)
33	8	5	398
		16	200
		50	141
		160	45
37	8	5	224
		16	200
		50	50
		160	32
33	16	5	447
		16	178
		50	126
		160	N.D.
37	16	5	1122
		16	250
		50	63
		160	50
33	24	5	251
		16	251
		50	126
		160	63
37	24	5	1122
		16	500
		50	398
		160	16

N.D. = not done.

pfu/cell and harvesting at 16 h p.i. (Table 4). Titres tended to decrease with increasing moi of S virus such that a moi of 160 reduced interference by 20 to 70-fold. The fact that DI virus titres vary with the moi of S virus may indicate that one DIP per cell is not sufficient to inhibit S virus synthesis by that cell, contrary to the suggestion by Sekellick and Marcus (180). There was little difference in titres obtained after 16 and 24 h incubation and results obtained at 33°C and 37°C.

c) Comparison of interference titres by YRA and RSIA

Interference by a number of DI SFV preparations was assayed by both the YRA and RSIA (Barrett et al., 1981). Both assays use L_{929} cells, the same number of cells, inoculum volume and temperature of incubation so allowing interfering properties to be directly compared. Reproducibility of the assays for interference by one DI SFV preparation (p5) is shown in Table 5. The mean and standard deviation (for explanation see Appendix) are shown for a number of measurements of the interference titre of DI SFV p5 by each assay. In statistical analysis it is usual to look at the standard error (see Appendix). Thus the reliability of a sample mean in indicating the true mean of the whole population can be described. It is also useful to attach confidence limits at a certain level of probability (usually 95%, see Appendix).

Therefore, in Table 5 the mean YRA interference titre lies between 38.9 and 53.8 DIU/ml, the mean RSIA interference titre lies between 28.5 and 38.3 DIU/ml and the ratio of the YRA to RSIA lies between 1.29 and 1.49.

Table 5 Reproducibility of interference titres of DI SFV p5 obtained by the YRA and RSIA

	Interference Titre (DIU/ml)*	
	YRA	RSIA
Range	50, 56, 45, 40, 28, 56, 50	28, 28, 32, 40, 28, 35, 50, 32, 28
Standard Deviation	9.9	7.4
Mean	46.4 ± 3.8†	33.4 ± 2.5
Confidence limits (95%)	38.9 to 53.8	28.5 to 38.3
Ratio‡	1.39 ± 0.05†	
Confidence limits	1.29 to 1.49	

* Each obtained on separate occasions

† One standard error of the mean

‡ All permutations of interference titres by the YRA/interference titre by RSIA were calculated

Clearly the two assays do not give the same interference titre for the DI SFV preparation.

These results were extended by comparing the interference titres by the 2 assays for 11 DI SFV preparations which had been passaged between 5 and 19 times at high multiplicity (Table 6). Two conclusions emerged, firstly, that the amount of interference measured by both assays varied between preparations and was not related to passage level. Secondly, there was no consistency in the ratio of the titre obtained by the YRA and the RSIA between preparations. When 95% confidence limits were applied to the interference titres in Table 6 the YRA:RSIA ratios of DI viruses p5, p6, p11, p13h, p17a, p17c and p18 were significantly different.

If the two assays measured the same interference property, the ratio of the titres (YRA:RSIA) should be constant from preparation to preparation. Instead the ratio varied up to 46-fold (p17a is 0.16 and p11 is 7.29) indicating that there are at least two types of interference mediated by DI SFV preparations. Therefore, it is concluded that DI SFV preparations are biologically heterogeneous and that their properties vary from passage to passage. However, sister preparations at passage 13 and passage 17 were relatively constant in interference titre and the ratio of YRA titre to RSIA titre varied by only 3-fold, indicating that variation was not random.

Table 6 Comparison of assays for measuring interference by DI SFV

DI SFV Preparation	Interference Titre (DIU/ml) YRA	RSIA	YRA:RSIA titre
p5 (B5)	46 ± 3.8	33 ± 2.6	1.40 ± 0.05
p6 (B6)	502 ± 19.8	343 ± 27.4	1.47 ± 0.04
p9 (B9)	22 ± 3.5	31 ± 6.0	0.69 ± 0.14
p11 (B11)	170 ± 20.6	23 ± 3.4	7.29 ± 0.70
p13d (B13)	103 ± 14.7	83 ± 6.1	1.24 ± 0.07
p13h (B13)	200 ± 0.0	66 ± 3.2	3.02 ± 0.09
p17a (B16 C1)	39 ± 6.5	235 ± 16.0	0.16 ± 0.02
p17b (B16 C1)	71 ± 8.0	143 ± 43.1	0.50 ± 0.10
p17c (B16 C1)	39 ± 6.5	187 ± 13.0	0.16 ± 0.02
p18 (B17 C1)	59 ± 4.3	36 ± 4.0	1.63 ± 0.11
p19 (B17 C2)	946 ± 54.7	1012 ± 219.6	0.93 ± 0.13

* All estimates of errors correspond to 1 standard error of the mean (see Table 5).

d) Sensitivity of the RSIA and YRA interference assays to UV irradiation

The evidence presented above suggests that the RSIA and YRA interference assays measure different parameters of interference. To explore this possibility further, the kinetics of UV inactivation of interference by DI viruses p13d and p20c were investigated (Figures 9a and 9b). The inactivation curves for both DI viruses show that interference is more resistant than infectivity to UV irradiation as has been described for a number of systems (VSV: Huang et al., 1966; Winship and Thacore, 1979; Bay and Reichmann, 1979; lymphocytic choriomeningitis virus: Welsh et al., 1972; Popescu et al., 1976; influenza virus: Nayak et al., 1978; SV: Kowal and Stollar, 1980; and SFV: Dimmock and Kennedy, 1978; Barrett et al., 1981). Both DI viruses were inactivated by single hit kinetics and for both p13d and p20c interference measured by RSIA was more resistant to inactivation.

From this data the following conclusions can be made:

i) It appears that a nucleic acid, and not a protein was the radiation sensitive target since interference was inactivated at 256 nm, and the amount of this emission would not affect protein. Therefore a functional RNA is required for interference in both assays. This also suggests that interference takes place intracellularly and not at the level of attachment.

ii) The inactivation curves for both S and DI virus are exponential, i.e. single hit kinetics. This indicates that there is one functional

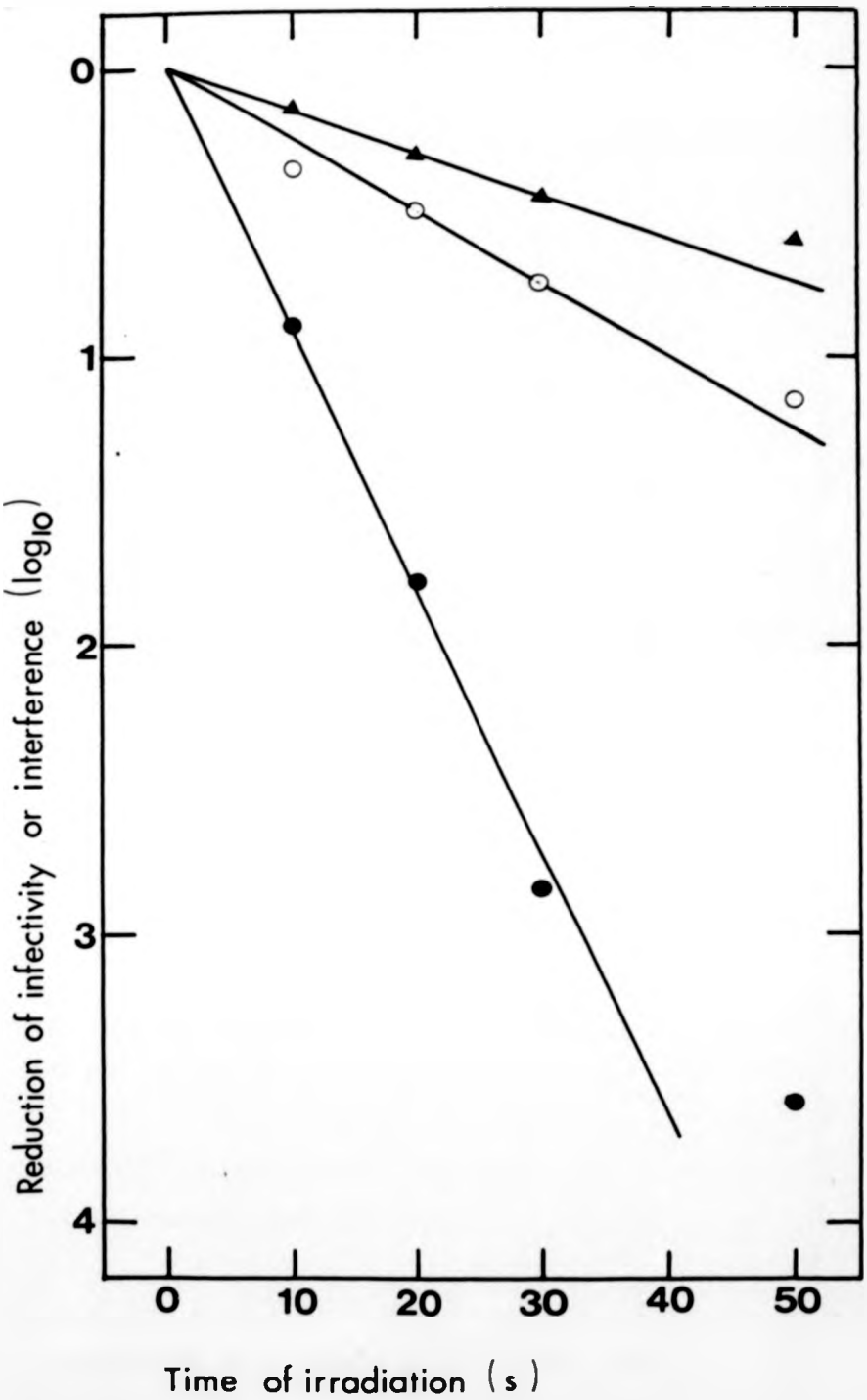

0
1
2
3
4
0
10
20
30
40
50
Reduction of infectivity or interference (log_{10})
Time of irradiation (s)

Figure 9a. UV inactivation curve of interference by DI SFV preparation p13d

1 ml aliquots were irradiated as described in Methods and titrated for infectivity (●) and interference by RSIA (o) and YRA (▲).

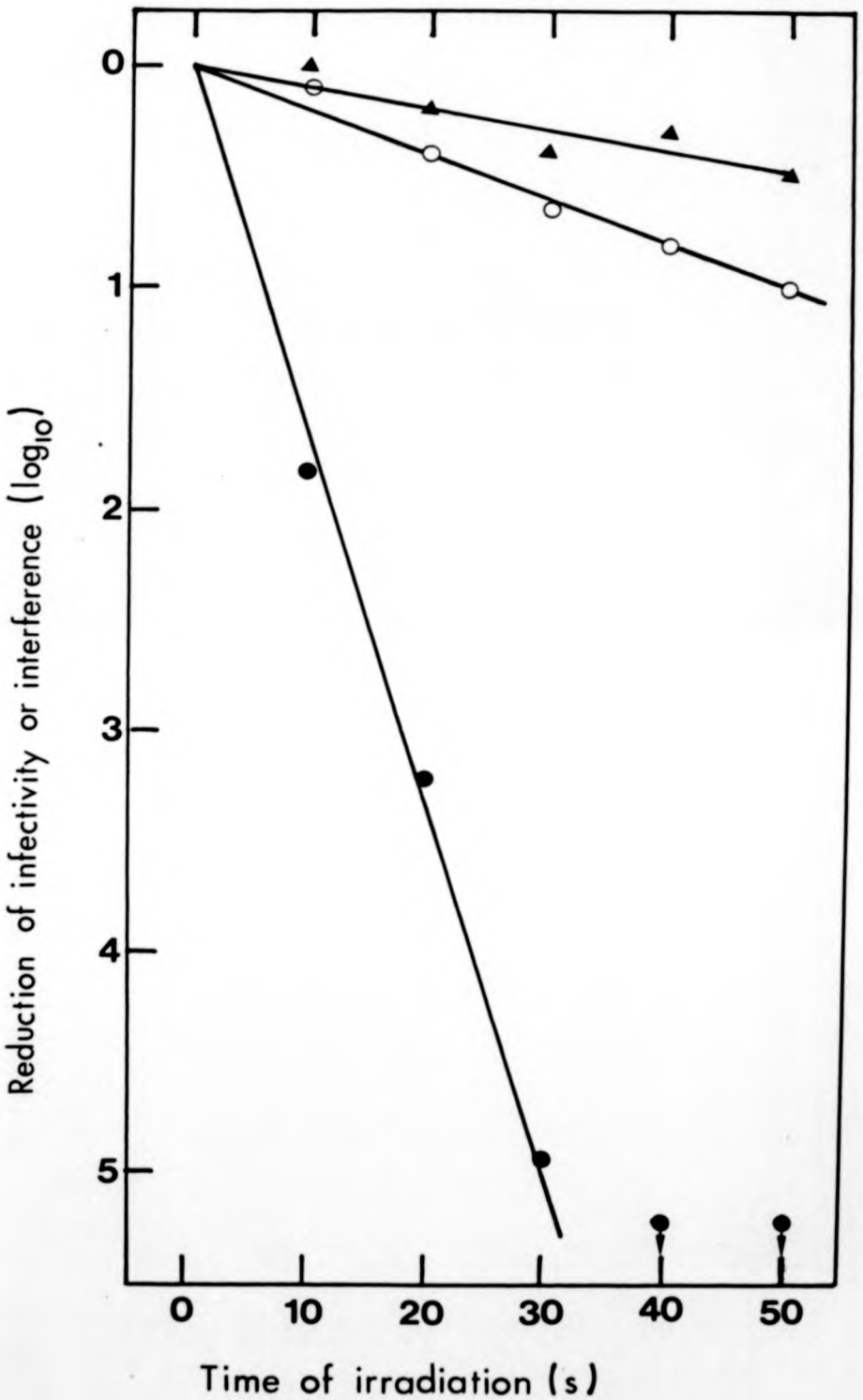

Reduction of infectivity or interference (log_{10})
0
1
2
3
4
5
0
10
20
30
40
50
Time of irradiation (s)

Figure 9b. UV inactivation curve of DI SFV preparation p20c

1 ml aliquots were irradiated as described in Methods and titrated for infectivity (●) and interference by RSIA (o) and YRA (▲).

nucleic acid per virus particle for S virus and DI virus as measured by both assays.

As mentioned above, interference measured by the RSIA was more resistant than that by the YRA implying that the size of the RNA required for RSIA is smaller.

The measurement of target sizes from UV inactivation data is based upon the failure of nucleic acid transcription to extend beyond the site of a UV lesion (Michalke and Bremer, 1969; Abraham and Banerjee, 1976; Ball and White, 1976). UV irradiation causes lesions on a RNA molecule by generating pyrimidine dimers at sites where these nucleotide residues are adjacent (Miller and Plagemann, 1974) and this results in premature termination of transcription (Hackett and Sauebier, 1975). The target size for interference by the RSIA and YRA can be determined from statistical analysis of the UV inactivation curve for interference and infectivity shown in Figures 9a and 9b (see Appendix). Therefore, for DI SFV p13d in Figure 9a the $t_{37\%}$ for infectivity is 9 sec; while for interference measured by the RSIA it is 54 sec and by the YRA 32 sec. Thus interference by RSIA requires the activity of 9/54 = 16.7% as much virus RNA as does plaque formation. Hence the relative sensitivity of interference by the RSIA is about 16.7% of infectivity. If it is assumed that the complete S virus genome is required for infectivity and the M_r of S virus RNA is 4.3×10^6 (Kääriäinen and Söderlund, 1978), the target size of the DI SFV p13d which causes interference by the RSIA is 16.7% of $4.3 \times 10^6 = 7.2 \times 10^5$. By similar calculations the other UV target sizes of interference for the two DI virus preparations can be

Table 7 UV target sizes of interference by RSIA and YRA for DI viruses p13 and p20

DI virus	UV target size of interference†	
	RSIA	YRA
p13	7.2×10^5	1.2×10^6
p20	2.7×10^5	5.4×10^5

† UV target size relative to S virus genome (4.3×10^6)

determined (Table 7). The UV target size of interference by the YRA is twice that of the RSIA for both DI viruses examined.

e) Estimate of M_r of DI SFV RNAs by gel electrophoresis

To physically size DI SFV RNA species [^{3}H]-uridine labelled RNA from purified DI virus preparations, p13 and p20, was extracted and sized on a formamide-agarose gel (Figure 10). The physical size of DI RNAs relative to chicken ribosomal RNA markers shows that both DI viruses have genomes closely similar in size.

f) Differential effects of DI virus on cellular and viral polypeptide synthesis in co-infected cells

The experiments described above have shown that DI virus preparations differ in their ability to interfere as measured by reduction in total virus RNA synthesis, yield of S virus. In this section the latter was studied in more detail by looking at the effect of DI virus on polypeptide synthesis in co-infected cells.

1) Virus RNA synthesis in cells co-infected with DI and S virus

These experiments were undertaken before investigating polypeptide synthesis to determine the extent to which DI virus inhibits and/or delays virus RNA synthesis and the time at which maximum interference is exerted. Cultures were labelled for 1 h with [^{3}H]-uridine to measure the rate of total virus RNA synthesis. Actinomycin D was present throughout infection so that the majority of [^{3}H]-uridine was incorporated into virus

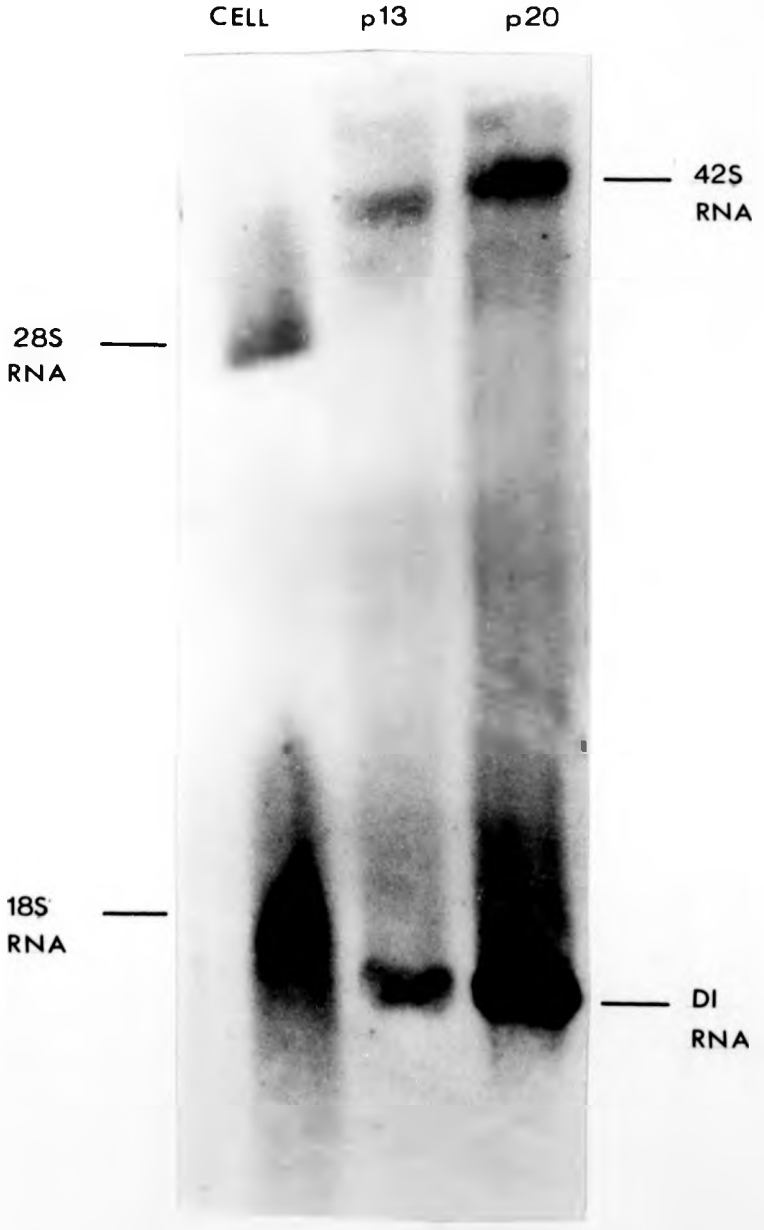

CELL
p13
p20
42S RNA
28S RNA
18S RNA
DI RNA

Figure 10. Agarose gel electrophoresis of virus RNA

RNA was extracted from extracellular virus and sized on an agarose gel. DI SFV preparation (p13), DI virus preparation (p20), ribosomal RNA (CELL).

RNA and to exclude possible effects of interferon. In the absence of added DI virus, RNA synthesis in infected L_{929} cells peaked at 5 h p.i. and decreased thereafter (Figure 11). On addition of DI virus at the time of infection, the resulting virus RNA synthesis was reduced but peaked at the same time as S virus infected cultures. Inhibition of virus RNA synthesis was proportional to the concentration of DI virus inoculated, cultures co-infected with the most concentrated DI virus had 7.2% of [^{3}H]uridine incorporation by S virus alone. Contrary to expectation DI virus did not delay virus RNA synthesis. DI virus interfered with RNA synthesis at all times during infection and the maximum effects were observed between 4 and 5 h p.i. (Figure 12).

2) Inhibition of cellular and SFV-specified polypeptide synthesis

In the following studies mouse L_{929} cells were used. This system differs from hamster (BHK) and chick (embryo fibroblast) cells in that the synthesis of non-structural polypeptides is not detected until 2 to 3 h p.i. Virus structural polypeptide synthesis and shut off of host protein synthesis are not detected until 3 to 4 h p.i. Virus RNA synthesis peaks at 4 to 5 h p.i. (Figure 11), so it appears that SFV replication takes place more slowly in L_{929} than BHK cells (Bruton and Kennedy, 1975; Clegg *et al.*, 1976).

The affects of three DI SFV preparations, p6, p12e and p20a, on polypeptide synthesis in co-infected cells were compared. Cultures infected with DI virus alone had a pattern of polypeptide synthesis indistinguishable from those in uninfected cultures and no DI virus-specified polypeptides were observed.

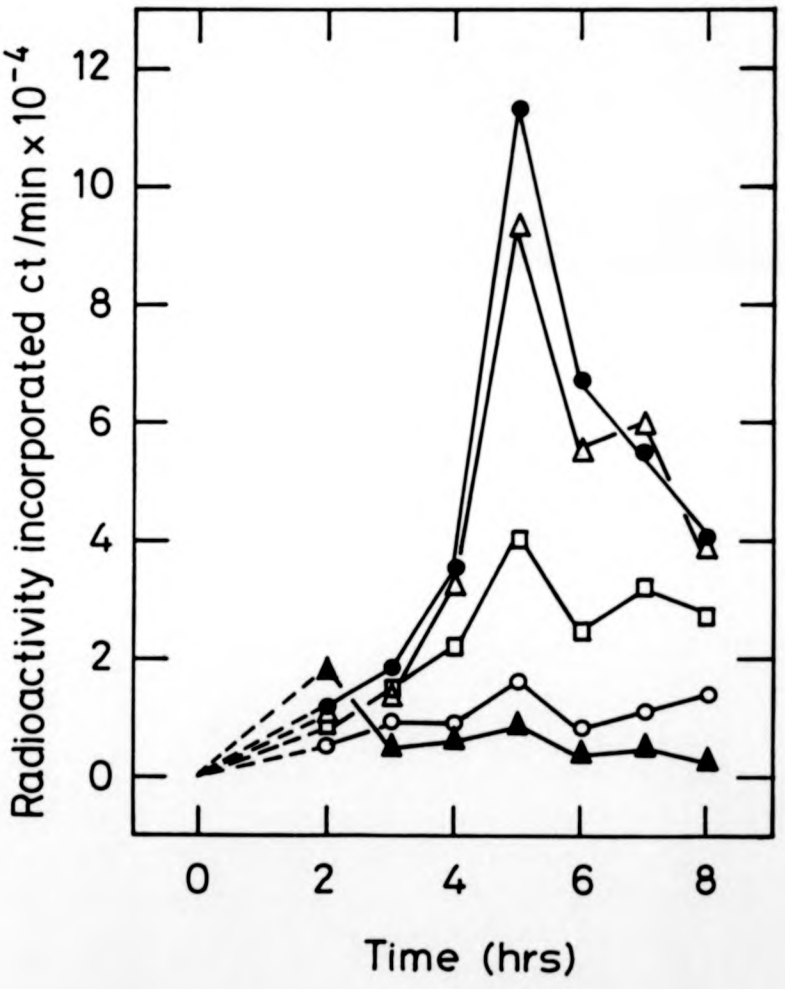

Radioactivity incorporated ct/min x 10⁻⁴
12
10
8
6
4
2
0
0
2
4
6
8
Time (hrs)

Figure 11. Virus RNA synthesis in L_{929} cells at different times after infection with S virus (moi of 50) + DI virus p13f or S virus only

Mock-infected cells ▲; S virus (moi of 50) infected cells ●; S virus (moi of 50) infected cells co-inoculated with 1/1 DI SFV o; with 1/10 DI SFV □; with 1/100 DI SFV Δ. Cells were pulsed for 1 h with [^{3}H]-uridine and harvested at the times indicated. Actinomycin D (2 µg/ml) was present throughout infection to inhibit cellular RNA synthesis.

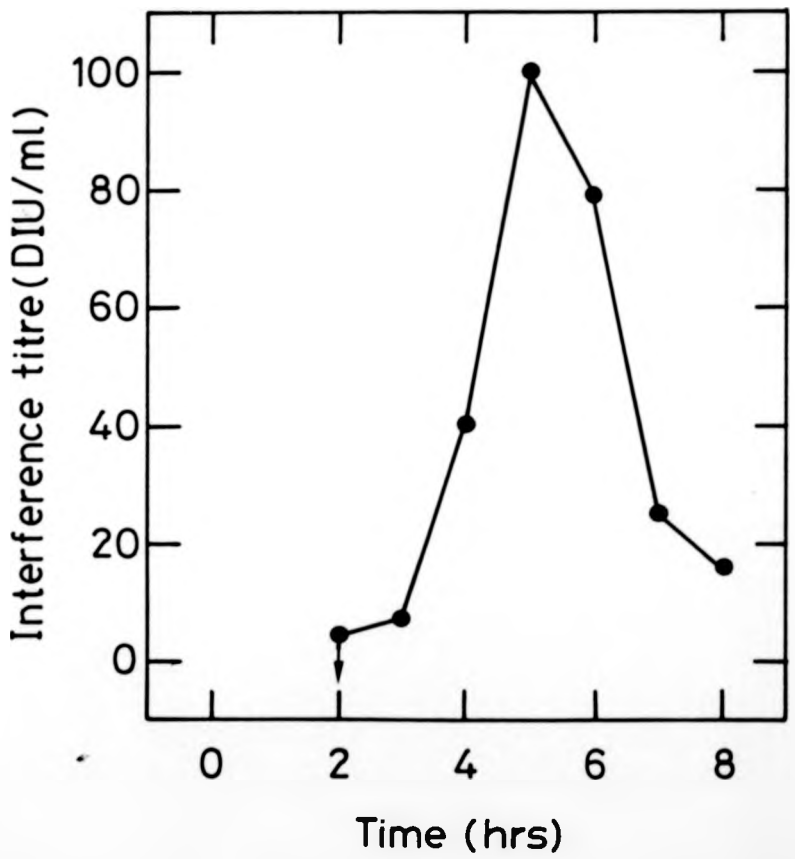

Interference titre (DIU/ml)
100
80
60
40
20
0
0
2
4
6
8
Time (hrs)

Figure 12. Determination of the time of maximum interference by DI SFV p13f

Interference was measured according to Barrett et al. (1981) by pulsing cells for 1 h periods with [^{3}H]-uridine throughout infection.

i) Inhibition of cellular and viral polypeptide synthesis by DI SFV p6 and p12e

In the first experiments cultures were co-infected with a constant multiplicity of S virus of 50 together with various concentrations of DI virus p12e and pulsed with [^{35}S]methionine from 4 to 5 h p.i. Analysis by PAGE showed that in cultures infected with the highest concentrations of DI virus p12e, the pattern of polypeptide synthesis was identical to that in mock-infected cultures (Figure 13a). Thus DI SFV p12e not only inhibits virus polypeptide synthesis but also the virus-induced inhibition of host protein synthesis. Dilution of DI virus ($10^{-0.5}$) again resulted in no detectable virus-specified polypeptides, but host protein synthesis was shut off. As DI virus was further diluted ($\geq 10^{-1}$) synthesis of virus structural polypeptides resumed and this increased inversely with the concentration of DI virus. Another DI virus, p6, shut off host protein synthesis and the synthesis of both virus structural polypeptides, and the non-structural polypeptides nsp63 and nsp90 (Clegg et al., 1976; Clewley and Kennedy, 1976; Logan, 1979) were inhibited (Figure 13b).

ii) Inhibition of cellular and viral polypeptide synthesis by DI SFV p20a

Figure 14 shows that DI SFV p20a, like p6 and p12e, inhibited the shut off of host protein synthesis, but DI virus p20a differed in that it failed to completely inhibit virus structural polypeptide synthesis. Although polypeptides E1 and E2 were undetectable at high concentrations of DI virus, there were significant amounts of the structural precursor

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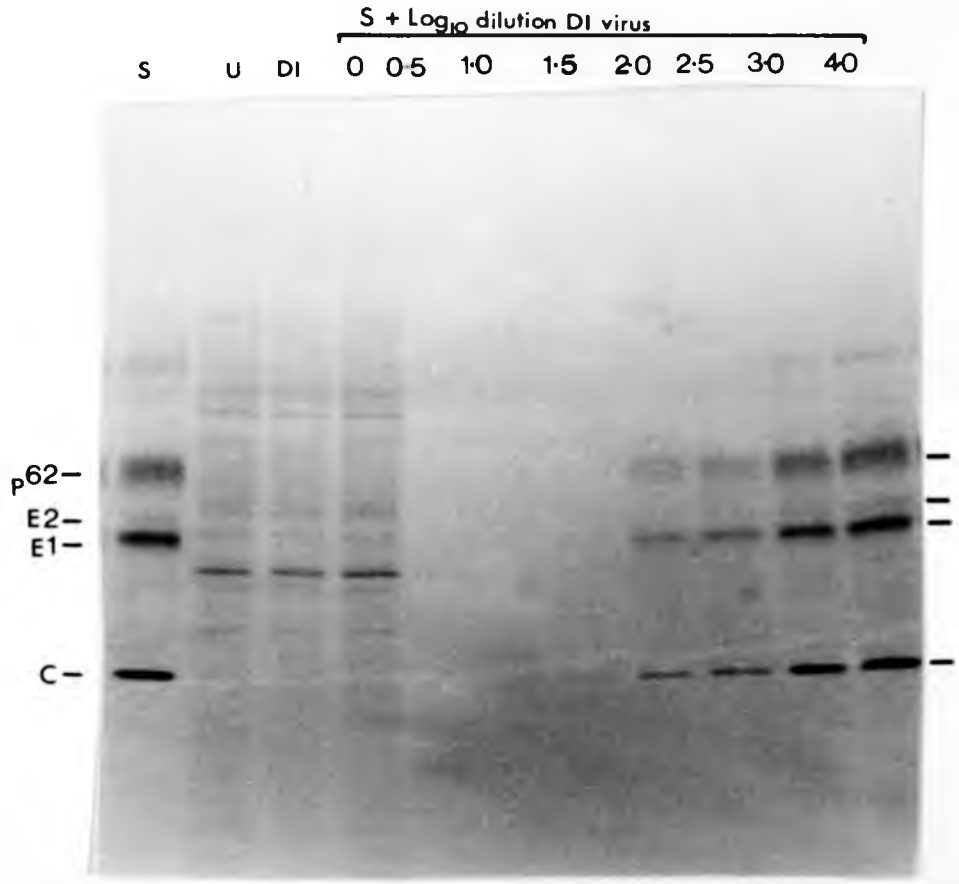

S + Log10 dilution DI virus
S
U
DI
0
0·5
1·0
1·5
2·0
2·5
3·0
4·0
p62
E2
E1
C

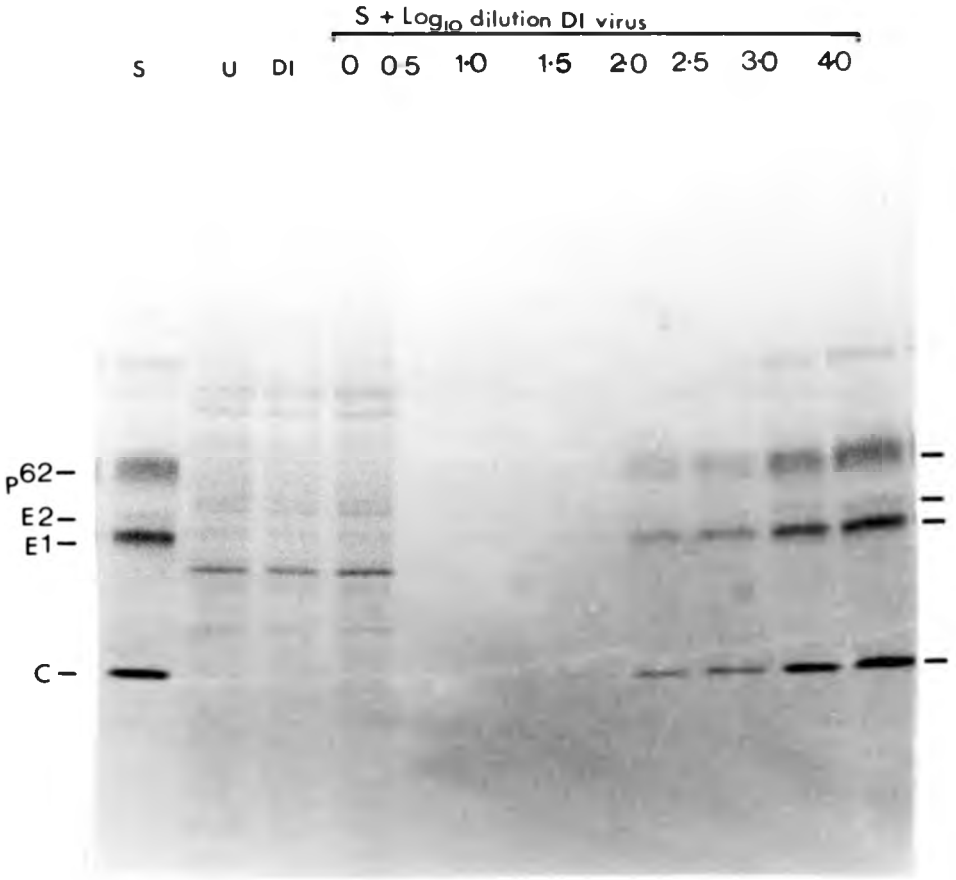
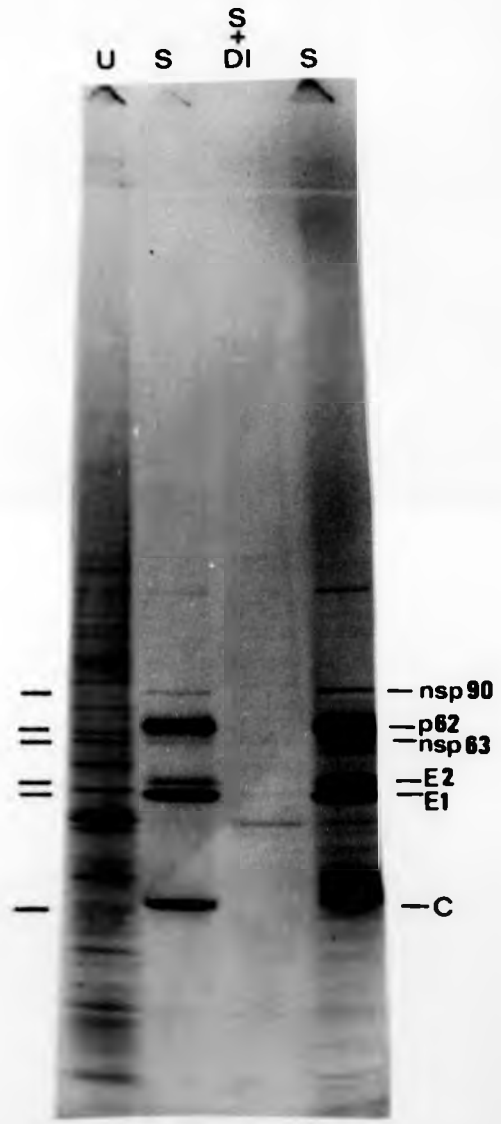

S + Log10 dilution DI virus
S
U
DI
0
0·5
1·0
1·5
2·0
2·5
3·0
4·0
p62
E2
E1
C

Figure 13a. SFV polypeptides synthesised in cultures co-infected with S virus and various concentrations of DI SFV p12c.

S virus (moi of 50) (S); uninfected cells (U); DI SFV p12c only (DI); S virus (moi of 50) plus various concentrations of DI SFV p12c (S + DI). Cultures were pulsed with [^{35}S]methionine from 4 to 5 h p.i.


U
S
S + DI
S
nsp90
p62
nsp63
E2
E1
C

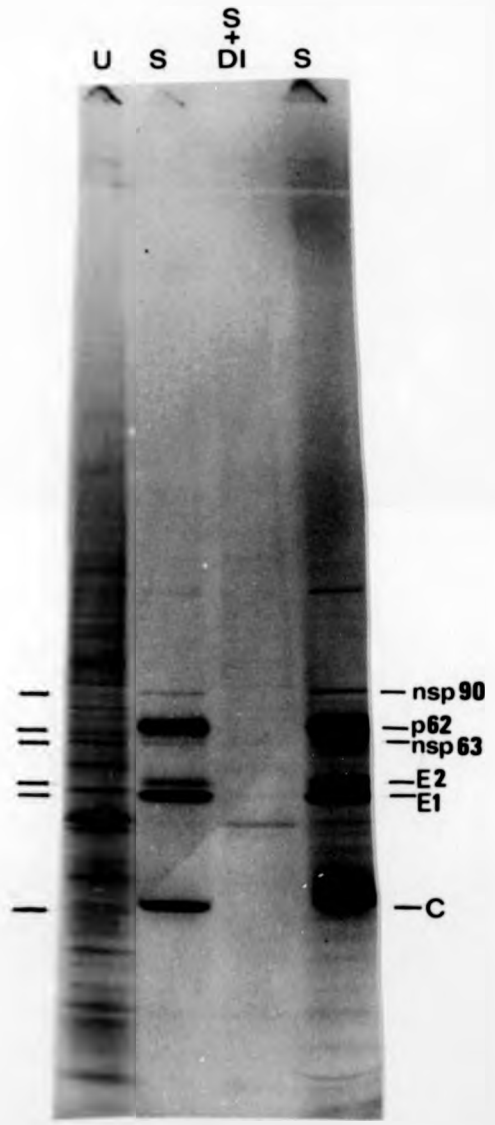
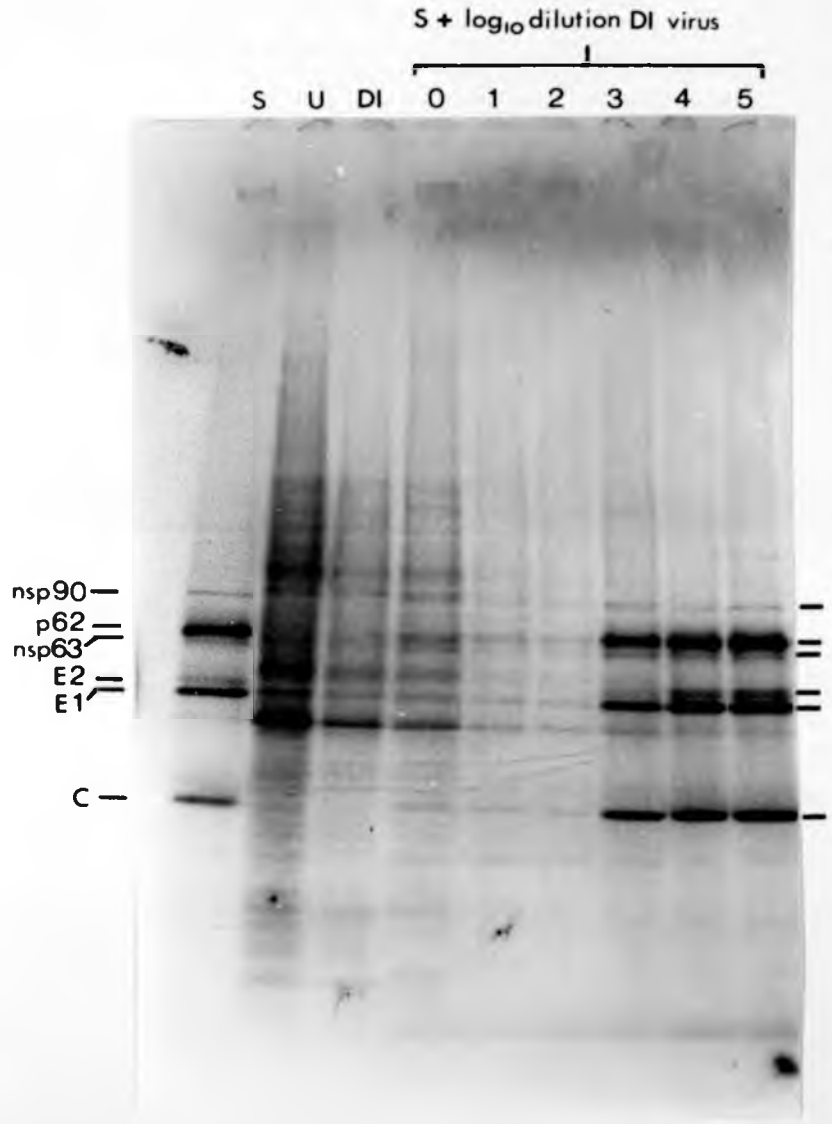

U
S
S + DI
S
nsp90
p62
nsp63
E2
E1
C

Figure 13b. Inhibition of non-structural polypeptide synthesis in cultures co-infected with S virus and DI SFV p6

Uninfected U; standard virus (moi of 50) (S); standard virus (moi of 50) plus DI SFV p6, (S + DI). Cultures were pulsed with [^{35}S]methionine from 4 to 5 h p.i.


S + log10 dilution DI virus
S
U
DI
0
1
2
3
4
5
nsp90
p62
nsp63
E2
E1
C

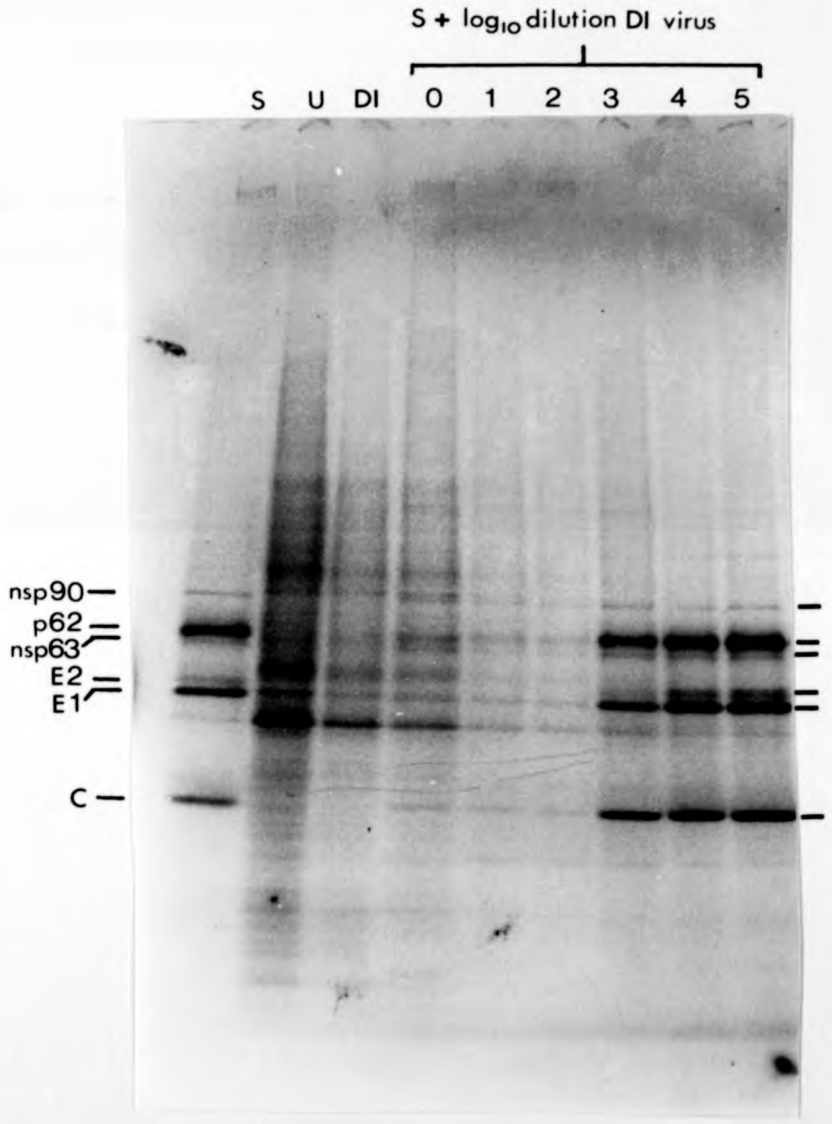

S + log10 dilution DI virus
S
U
DI
0
1
2
3
4
5
nsp90
p62
nsp63
E2
E1
C

Figure 14. Polypeptides synthesised in cultures co-infected with S virus and different concentrations of DI SFV p20a

Standard virus (moi of 50) (S); uninfected cells (U); DI SFV p20a only (DI); Standard virus (moi of 50) plus various concentrations of DI SFV p20a (S + DI). Cultures were pulsed with [^{35}S]methionine from 4 to 5 h p.i.

p62 and core polypeptides present throughout. This contrasts with the data in Figures 13a,b where virus polypeptide synthesis is seen only after host protein synthesis has been inhibited.

Inspection of Figure 14 showed that there was significant synthesis of the non-structural polypeptides in the presence of DI virus p20a, a marked difference to the effects of DI virus p6 (Figure 13b). However, quantitation of radioactivity (Figure 15) showed that their synthesis had been affected unequally. In particular, synthesis of nsp90 was barely affected and there was synthesis of significant amounts of nsp63 even in the presence of the most concentrated (10^0 to 10^{-2}) samples of DI virus. Compared with S virus there was considerable variation in the relative amounts of virus polypeptides synthesised in cultures co-infected with high concentrations of DI virus (10^0 to 10^{-2}). On further dilution of DI virus the ratio of virus polypeptides returned to that seen in cultures infected with S virus alone.

Apart from nsp90, inhibition of virus polypeptide synthesis showed a linear response over a 1000-fold range of DI virus dilution. The 50% inhibition point was very reproducible occurring between $10^{-3.2}$ and $10^{-3.5}$ (Figure 15). Under identical conditions (Barrett *et al.*, 1981) the 50% inhibition of virus RNA synthesis (RSIA) occurred at $10^{-2.0}$, the difference presumably reflecting an amplification resulting from repeated translation of mRNAs.

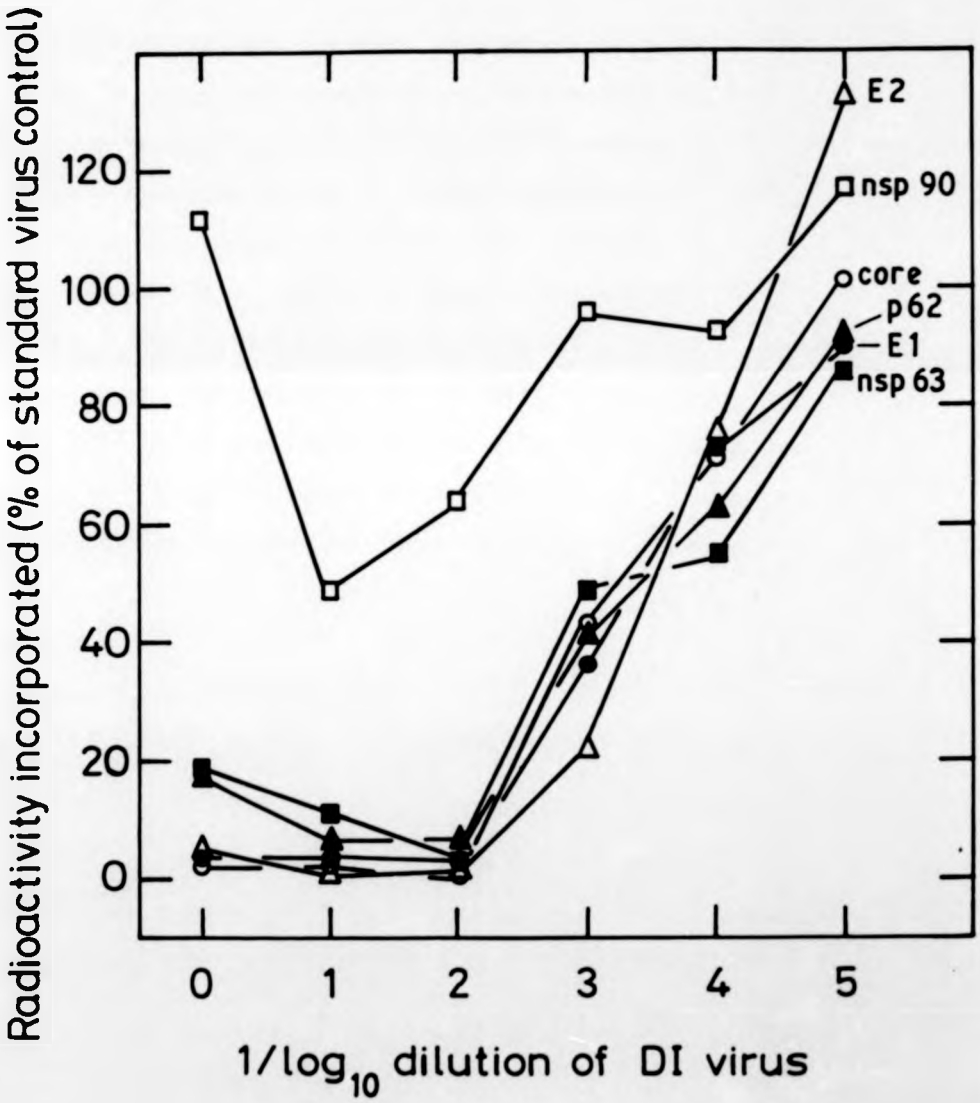

Radioactivity incorporated (% of standard virus control)
120
100
80
60
40
20
0
0
1
2
3
4
5
1/log10 dilution of DI virus
E2
nsp 90
core
p62
E1
nsp 63

Figure 15. Radioactivity incorporated into virus polypeptides synthesised in cultures co-infected with S virus and different concentrations of DI SFV p20a

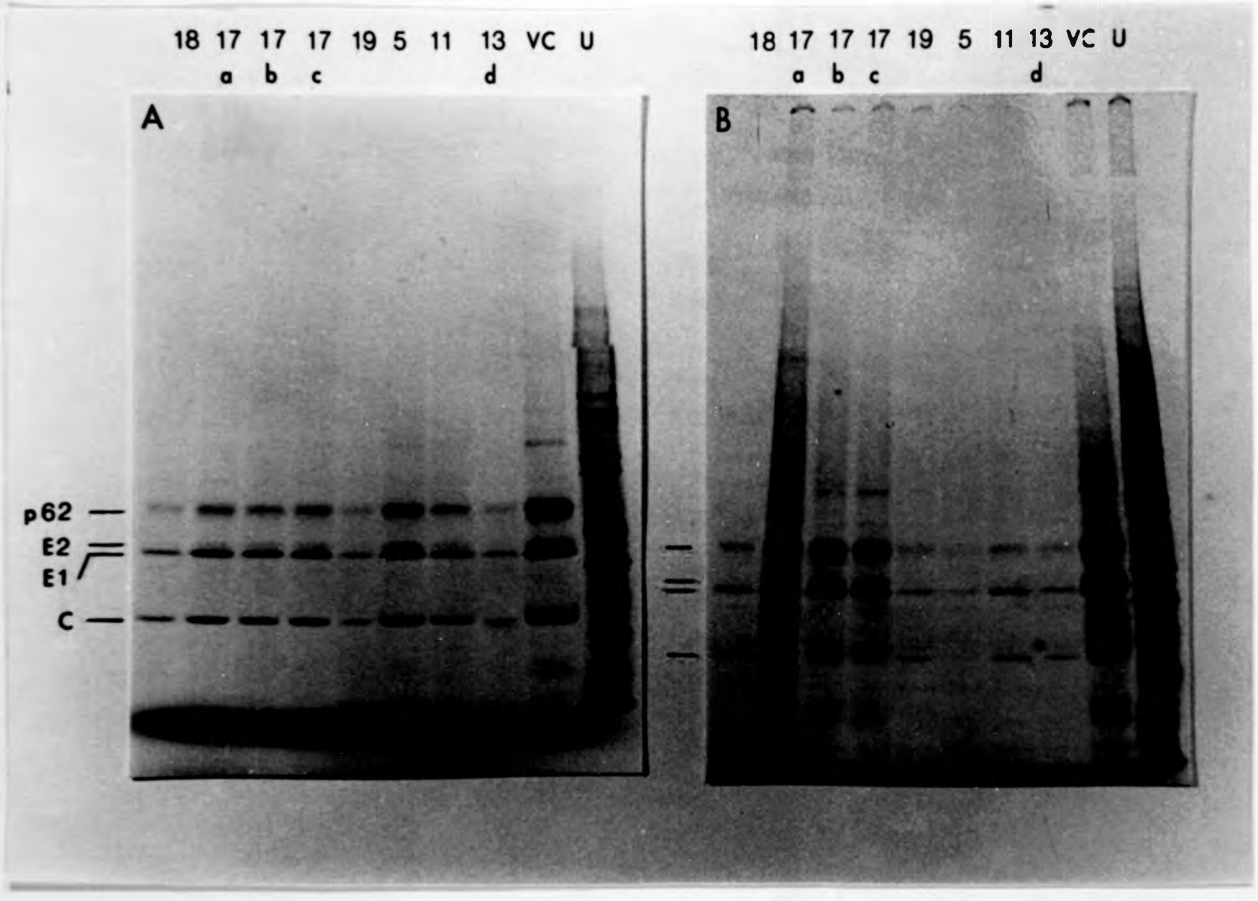
Polypeptides in Figure 14 were excised from the gel and the radioactivity determined as described in Methods. Core o; E1 ●; E2 △; p62 ▲; nsp90 □; nsp63 ■.

g) Propagation of DI virus at constant multiplicity

Interference by 8 DI SFV preparations was further investigated by measuring three parameters of interference (inhibition of virus polypeptide synthesis, inhibition of infectious virus production and synthesis of DI virus) in parallel. Each DI virus preparation was inoculated onto L_{929} cells at a constant interference titre (4 DIP/cell) based on the value obtained by the YRA or the value obtained by the RSIA. Conditions were the same as those of the YRA and RSIA except that a moi of 50 pfu/cell was used to obtain sufficient virus polypeptide synthesis.

1) Virus polypeptide synthesis

Polypeptide synthesis in cells inoculated with DI SFV standardised by the YRA is shown in Figure 16A. The major virus-induced polypeptides (p62, E1, E2, C) were excised and radioactivity in each was determined. The synthesis of all polypeptides was equally depressed and the synthesis of individual polypeptides was expressed as a percentage of that synthesised by cells inoculated with standard virus alone. These values were summed and the means presented in Table 8. Most DI virus preparations reduced standard virus polypeptide synthesis to 10-15% of the normal value, but DI viruses p19 and p13d allowed only 3 and 4% synthesis respectively. DI virus p5 inhibited less efficiently (30% synthesis). DI virus inocula standardised by the RSIA showed greater variation in polypeptide synthesis (Figure 16B), while most (5/8) inhibited virus polypeptide expression to below 4%, the others (3/8) allowed 27 to 35% synthesis (Table 8). Clearly there was no correlation between interference titre of the inoculum DI virus and its ability to


18 17 17 17 19 5 11 13 VC U
a b c d
A
p62
E2
E1
C
18 17 17 17 19 5 11 13 VC U
a b c d
B

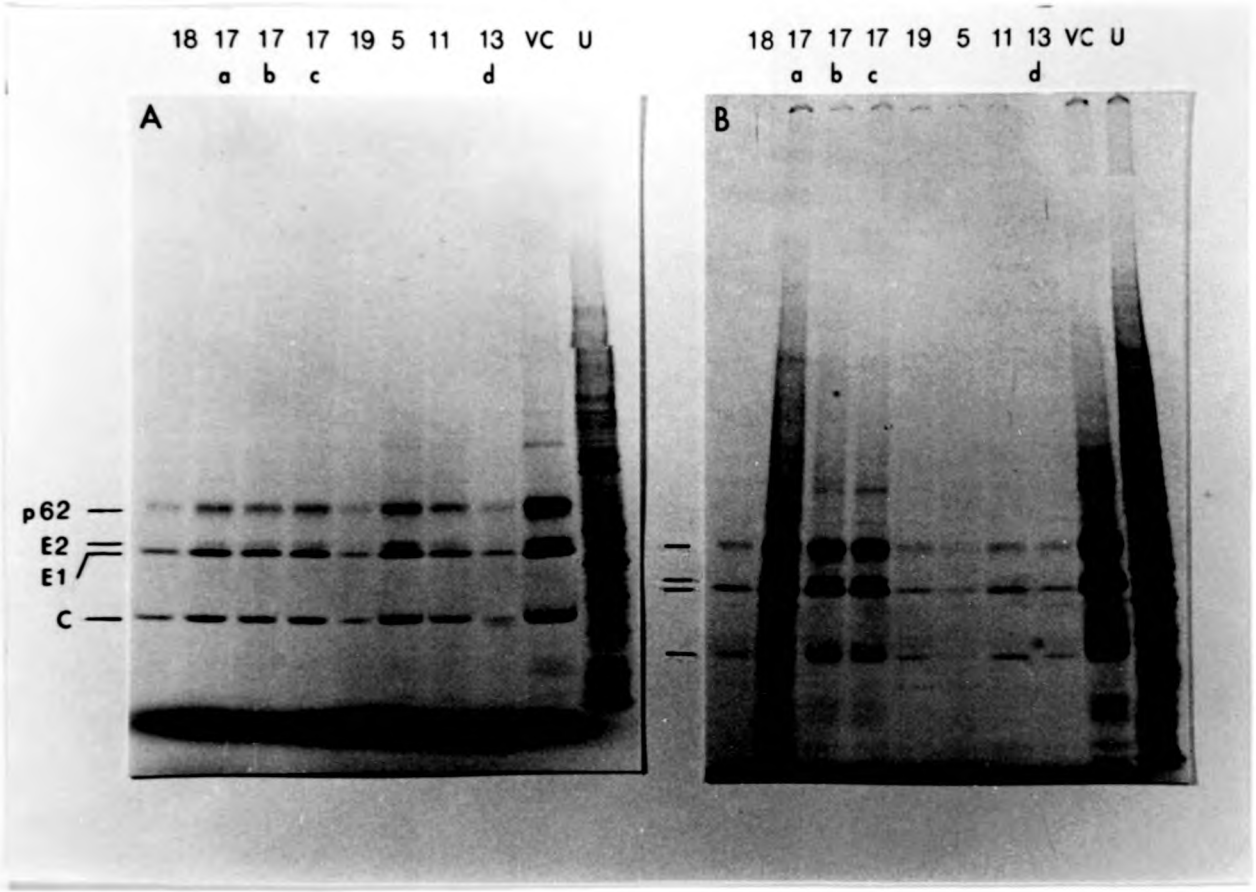

18 17 17 17 19 5 11 13 VC U
a b c d
A
p62
E2
E1
C
18 17 17 17 19 5 11 13 VC U
a b c d
B

Figure 16. Polyacrylamide gel electrophoresis of virus polypeptides synthesised in L_{929} cells inoculated with a constant multiplicity of DI virus and S virus.

Panel A: cultures inoculated with 4 DIP/cell as determined by the YRA. Panel B: cultures infected with 4 DIP/cell as determined by the RSIA. In addition all virus infected cultures received S virus (moi of 50). VC = cultures infected with S virus (moi of 50) only; U, uninfected cultures. Tracks were loaded with equal numbers of cells as determined by [^{3}H]-thymidine labelling.

Table 8 Parameters of interference in L_{929} cells inoculated with DI SFV and standard virus

Inoculum DI SFV*		Yield from cells inoculated with 4 DIP/cell as titrated by the YRA					Yield from cells inoculated with 4 DIP/cell as titrated by the RSIA				
Passage number	Titre† YRA /RSIA	Virus polypeptide synthesis %	p.f.u. %	DIU/ml YRA	DIU/ml RSIA	YRA /RSIA	Virus polypeptide synthesis %	p.f.u. %	DIU/ml YRA	DIU/ml RSIA	YRA /RSIA
p5	1.4	30	3	16	13	1.2	2	5	25	50	0.5
p11	7.3	10	7	⩽4	40	⩽0.1	6	6	6	32	0.2
p13d	1.2	3	3	32	40	0.8	3	3	32	80	0.4
p17a	0.2	13	6	100	10	10.0	27	20	500	158	3.2
p17b	0.5	15	7	355	63	5.6	35	23	40	20	2.0
p17c	0.2	12	23	45	13	3.5	35	55	32	50	0.6
p18	1.6	11	94	32	126	0.3	3	74	10	63	0.2
p19	0.9	4	1	22	126	0.2	4	2	63	50	1.3
SFV	N.A.	100	100‡	⩽4	⩽4	N.A.	100	100	⩽4	⩽4	N.A.

* Cells were also inoculated with 50 p.f.u./cell

† Titres are shown in Table 6

‡ 6.4×10^8 p.f.u./culture

N.A. not applicable

This table represent a single experiment; a duplicate gave good reproducibility.

affect the synthesis of virus polypeptides. None of the DI viruses specified the synthesis of any novel protein.

All DI virus preparations shut off host cell protein synthesis except p17a (Figure 16B) even though it was less efficient than the others at inhibiting virus polypeptide synthesis. It seems, therefore, that the expression of virus polypeptides does not necessarily cause the inhibition of host cell protein synthesis, and this is at odds with current theories (Wengler, 1980). Another DI SFV preparation, p20a, also failed to shut off host protein synthesis (see Figure 14).

2) Yield of infectious virus

Most (6/8) DI virus preparations standardised by the YRA and half of those standardised by RSIA reduced production of infectious virus to 7% or less (Table 8). The notable exception was DI virus p18 which gave titres of infectious progeny of 74% or more relative to standard virus, although polypeptide synthesis was greatly decreased (to about 10%) and considerable amounts of DI virus were synthesised. Other DI virus preparations gave intermediate levels of interference. The apparent inability of DI virus p18 to interfere with virus production, when it clearly did so in the YRA assay shown in Table 6, may have been the result of using a moi of 50 pfu/cell rather than 5 pfu/cell in the YRA assay. Table 9 confirms this suggestion and demonstrates that another DI virus (p13d) interfered equally well at a high or low moi.

3) Yield of DI virus

Yields of DI virus varied upto 100-fold when measured by the YRA and

Table 9 Interference titres by the YRA in L_{929} cells infected with standard virus at different multiplicities of infection

DI virus	DIU/ml	
	m.o.i. = 5	m.o.i = 50
p13d	80	126
p18	64	$\leqslant 4$

upto 10-fold by the RSIA (Table 8). Cells inoculated with DI virus preparation which had a high YRA titre relative to the RSIA titre, gave either progeny with a similar ratio of interference titres or with the ratio reversed. For example, DI virus p17b yielded progeny DI virus which gave interference which registered predominantly by the YRA whereas p11, inoculated at the same YRA titre, gave interference detectable only by the RSIA.

In summary, cells inoculated with a standardised interfering dose of DI SFV of different passage history showed different parameters of interference. These varied without respect to the interfering titre of the inoculum: thus cells inoculated by DI virus standardised by the YRA:DI virus p19 caused the greatest inhibition of infectious progeny (99%), a low yield of DI virus by the YRA and a high yield by the RSIA; DI virus p18 gave a similarly high yield of DI virus by the RSIA, reduced polypeptide synthesis by 89% and hardly affected the production of infectious progeny; lastly DI virus p13d inhibited virus polypeptide synthesis the most efficiently, reduced infectious progeny by 97% but gave a small yield of DI virus by either assay. The exception to this extreme variation were the sister stocks of p17 which behaved similarly, although host protein synthesis was less inhibited in the presence of p17a. Thus it seems that extensive differences appear on passage, but in the short term, the inoculum determines the biological properties of the progeny DI virus. Possible explanations will be discussed later.

h) Variation in homotypic and heterotypic interference

DI viruses were generated for one avirulent (A774) and two virulent (L10 and ts^+) strains of SFV (see Methods) and assayed for interfering activity by RSIA using S virus from each of the three SFV strains. The results (Table 10) show that avirulent (AV) DI virus p1 interfered with the replication of AV S virus and gave no detectable interference with either of the virulent strains. L10 DI virus p1 interfered with all three S viruses equally and ts^+ DI virus p19 interfered strongly with L10 and ts^+ S viruses but weakly with AV S virus. These results show that DI viruses derived from different strains of SFV have the ability to differentially interfere with the various S viruses, thus some mechanism must operate which allows discrimination in the interference event.

These observations were extended by investigating the stability of the observed spectrum of interference activity after one further passage, and by comparing interference with SV, another alphavirus which is serologically distinct but has a similar molecular biology (Table 11). Interference by ts^+ DI virus was unchanged upon passage and the DI virus demonstrated strong heterotypic interference with SV. After the additional passage L10 DI virus no longer interfered equally with the other SFV strains, showing reduced interference against AV S virus and none with ts^+ S virus. L10 DI virus p2 also interfered with Sindbis S virus. The second passage of AV DI virus interfered with all four S viruses equally in contrast to the previous passage which only interfered in the homologous reaction. Sindbis DI virus p2 gave strong homologous interference, reduced interference with L10 and ts^+ and none

Table 10 Interference measured by the RSIA between DI and S viruses of three strains of SFV

DI virus	Standard Virus		
	AV	L10	ts$^+$
pl AV†	50*	$\leqslant$4	$\leqslant$4
pl L10	13	10	7
pl ts$^+$	12	50	79

* Interference titre (DIU/ml)

† AV is avirulent strain A774

L10 is virulent strain L10

ts$^+$ is virulent strain ts$^+$

Table 11 Interference measured by RSIA of different strains of SFV DI virus after one further passage and interference by Sindbis DI virus

DI virus	Standard virus			
	AV	L10	ts$^+$	Sindbis
p2 AV†	16*	16	14	20
p2 L10	6	14	⩽4	8
P20 ts$^+$	63	501	501	562
p2 Sindbis	⩽4	18	10	100

* Interference titre (DIU/ml)

† AV is avirulent strain A774
L10 is virulent strain L10
ts$^+$ is virulent strain ts$^+$

with AV S virus. There was no detectable interference between the various combinations of different S viruses indicating that the results obtained above were due to DI virus-mediated interference. Interferon was not involved in the interference phenomena since actinomycin D was present throughout the RSIA. Thus, the specificity of interference between DI viruses of different strains of SFV can vary on passage. This confirms the results of experiments described above showing that the interference properties of DI SFV vary upon passage.

Discussion

Evidence has been presented that DI SFV preparations are biologically heterogeneous and that each parameter of interference varies on passage. Further, it is suggested that the putative different interfering elements within a DI virus population interfere at more than one step in the SFV replication cycle. Interference titres obtained by the YRA and RSIA show that some preparations have a higher titre by one assay than another and in others the ratio is reversed. It appears that the two assays measure independently varying parameters of interference acting at the level of virus RNA synthesis (RSIA) and at an unknown level (YRA). UV inactivation kinetics confirmed this theory and showed that the two parameters of interference had different sensitivities to UV irradiation. Another assay could be devised based on the inhibition of virus protein synthesis (Figures 15 and 16) and this again would represent an independant variable. The practical implication of these

findings is that it is impossible to passage DI SFV and to retain the properties of the parent (Table 8). Thus one is limited to working with apparently unrelated batches of DI virus. Fortunately though, sister stocks appear to be very similar.

The YRA and RSIA indicate that the extent of interference by a particular DI virus preparation depends on the ratio of DI SFV:S SFV per cell since the extent of interference decreases as infectivity is increased (Table 4), but there are exceptions to this generalisation (Table 9). This contrasts with Sekellick and Marcus (1980) who concluded that the presence of one DI particle/cell abrogated the production of S virus. A second point made in Table 8 is that interference (measured by several parameters) by different preparations of DI virus in cells inoculated with apparently the same amount of DI virus is variable.

Examination of the UV inactivation kinetics of interference showed that interference as measured by RSIA was more resistant than YRA to UV irradiation for both DI viruses examined (Figure 9). This would support the proposal that the two assays are measuring different parameters of interference. Since UV inactivation of interference took place with single hit kinetics it would appear that there is only one functional DI RNA genome per virus particle.

The UV target size for interference by two DI viruses was larger by YRA than for RSIA. This suggests that only part of the DI virus genome is required for interference at the level of RNA synthesis, while all the

genome is required for interference with the production of S virus progeny. This may indicate that DI RNA competes with S virus RNA at the level of virus assembly and this possibility will be considered later. Similar results have been reported by Kowal and Stollar (1980), which have shown for SV that the UV target size of interference (measured by YRA) was similar to the physical size of the DI virus genome for virus grown in BHK cells, but smaller than that of DI SV derived from mosquito cells. Also, Bay and Reichmann (1979), working with VSV, have obtained a DI virus with a UV target size smaller than the physical size of the genome. Sequence studies on SFV DI RNA by Lehtovaara et al. (1981, 1982) have shown that DI RNA consists of repeat units and it may be that the UV target size of interference by the RSIA corresponds to repeat unit(s) of the DI RNA for DI virus p20c. This possibility is supported by a repeat unit of 484 nucleotides (1.6×10^5) for DI SFV clone pKTH301 (Lehtovaara et al., 1981) a figure similar to the UV target size of interference by the RSIA for DI virus p20c.

The results of interference between different strains of SFV showed that DI SFV is capable of homotypic and heterotypic interference as measured by RSIA which varied upon passage (Tables 10 and 11). These findings are contrary to the general opinion that interference by DI virus is specific to the homologous S virus although DI particles of the Indiana serotype (HR DI 0.46) of VSV can interfere with S virus of the New Jersey serotype (Prevec and Kang, 1970; Schnitzlein and Reichmann, 1976). These observations give further support to the view that interference by DI SFV preparations varies on passage (Table 8) and also the specificity of interference varies on passage, be it at the level of

ratio of YRA:RSIA or homotypic interference.

Examination of the effects of DI virus on virus-polypeptide synthesis in co-infected cells also showed that different DI virus preparations behaved differently. Co-infection with the highest concentrations of DI virus preparations p6 and p12e abrogated the shut off of host protein synthesis and no virus protein synthesis was detectable. Progressive dilution of DI virus led successively to shut off of host cell protein synthesis, without virus protein synthesis, and then the appearance of virus structural polypeptides. This suggests that virus RNAs and not proteins are responsible for inhibition of host protein synthesis in infected cells, and this has also been suggested by Wengler (1980). However, co-infection of cultures with high concentrations of DI SFV p20a resulted in the limited synthesis of certain virus-specified polypeptides without shut off of host protein synthesis. A similar phenomenon was observed with DI SFV p17a (Figure 16B). This situation differs from infection of cells with S virus where shut off of host protein synthesis preceeds synthesis of virus structural proteins. The differential inhibition of nsp90 and nsp63 is difficult to interpret since little is known of their synthesis and their relationship to the non-structural polypeptides reported by other groups is uncertain. The reason for the difference in interference at the level of polypeptide synthesis between DI virus preparations p6/p12e and p20a is unknown.

Inhibition of virus polypeptide synthesis was 20-fold greater than virus RNA synthesis as would be expected from the amplification which occurs on translation of the mRNA. This extensive inhibition of virus-

specified polypeptides could be used as a sensitive assay for DI virus-mediated interference but the time needed to perform the assay makes it of little use in routine work. However, this indicates that the DI virus preparation used contains 20-fold more biologically active interfering particles than had been previously calculated by the RSIA (Barrett et al., 1981).

The reduced synthesis of both virion RNA and S virus polypeptides by co-infection with DI virus probably both contribute to the reduction in numbers of progeny virus particles. Presumably DI and S virus RNA will compete for a limited amount of structural proteins available for assembly of progeny virus particles. Therefore it can be envisaged that interference by DI SFV takes place not only during RNA synthesis, but also during assembly of virus particles. These two processes could be involved in the reduction in progeny S virus measured in the YRA since it is conceivable some DI RNAs interfere efficiently or poorly with virus RNA synthesis (RSIA), and then the DI RNAs may also be encapsidated efficiently or poorly (YRA?). The results of Kääriäinen et al. (1981) may support this theory (see below).

Synthesis of non-structural polypeptides was inhibited by co-infection with DI virus p6. Since these form part of the virus polymerase (Clewley and Kennedy, 1976) it would appear that virus polymerase activity is reduced in co-infected cultures. Less polymerase would result in a reduction in the total amount of virus RNA synthesised as observed (Guild and Stollar, 1975; Barrett et al., 1981; and Figure 11). Reduction in synthesis of non-structural polypeptides, may result

from inhibition of translation from the 42S genomic RNA of the infecting virus or, more likely, from competition between DI RNA and the input S virus RNA for newly synthesised polymerase. In contrast, there was little inhibition of synthesis of non-structural polypeptides after co-infection with DI virus p20a, suggesting that polymerase activity is not inhibited by all DI SFV preparations. Thus, DI virus mediated interference of S virus replication should not be thought of as "interference at the level of RNA synthesis", but rather interference at the level of polymerase synthesis, RNA synthesis and virus assembly. The results described above suggest that the extent of interference at each step will depend on the DI SFV preparation. This theory is supported by Kääriäinen et al. (1981) who have observed that some DI RNA species are replicated efficiently, whereas others are not; then some DI RNA species are encapsidated efficiently, and again others are not. It is clear from the studies described above that DI SFV preparations are heterogeneous in their ability to interfere and vary with each parameter examined.

Chapter 2

Protection of Mice by DI SFV

Introduction

In mice, virulent strains of SFV cause a lethal encephalitis after i.n. inoculation and in 1978 Dimmock and Kennedy reported that administration of DI SFV p8 prevented disease and death. Co-inoculation of DI SFV p8 plus 10 LD_{50} S SFV was necessary for maximum protection; inoculation of DI virus before or after infection resulted in a reduction in the protection. Optimally DI virus was given as two inoculations, 2 hours apart, with the second containing 10 LD_{50} S SFV. In this way the majority (50-90%) of DI SFV p8 treated mice were protected. It is interesting that protection was achieved with non-purified tissue culture fluid containing only 2 x 10^5 "pfu equivalents" of biologically active DI SFV particles whereas most other systems (see General Introduction) have involved high concentrations of purified DI virus. Controls showed that protection was not due to the immunogenic load of virus inoculated into the mice. Dimmock and Kennedy (1978) also presented evidence that DI virus was able to interfere with S virus *in vivo* and reduce multiplication of S virus in the brain by at least 10^5 fold. Cell culture amplification assays also showed the presence of DI virus in the brains of protected mice. A recent study by Crouch *et al.* (1982) supported the results of Dimmock and Kennedy (1978) and showed that the brains of mice protected by DI SFV p8 had no histological or histochemical pathology whatsoever.

In the previous chapter, DI SFV preparations were shown to be biologically heterogeneous in their ability to interfere *in vitro*. To date all the *in vivo* studies with DI SFV have involved DI SFV p8,

therefore the ability of a number of different DI SFV preparations to protect mice against 10 LD_{50} S SFV was examined.

Results

a) Determination of a sub-immunogenic dose of SFV antigen

In order to analyse the modulation of SFV infection in mice by DI SFV it was necessary to distinguish between the intrinsic interfering properties of the DI virus and its effects as an immunogen. Consequently, preliminary experiments were carried out to determine the threshold of immunisation for SFV antigen inoculated by the i.n. route. The amount of antigen was measured by haemagglutination (Clarke and Casals, 1958) and all DI SFV preparations tested were found to contain <4 HAU/ml (Table 13). Using a non-infectious non-interfering antigen, prepared by UV-irradiating S SFV, it was found that mice given two inoculations of 20 µl of a preparation containing 4HAU/ml 2 h apart, did not resist challenge by 100 LD_{50} S SFV administered i.n. 21 days later (Table 17). Consequently this amount of virus antigen could be used in the inoculum without the complication of immunogenic effects.

b) Protocol for protection experiments

As an internal control for immunogenic effects of DI virus, control mice were inoculated with UV inactivated S virus (UV SFV) diluted to 4 HAU/ml. Inoculation of UV SFV together with 10 LD_{50} S virus served also to control the possibility that DI virus was preventing access to cell

receptor sites. Table 12 shows this did not occur as the same number of mice died after inoculation with 10 LD_{50} only or 10 LD_{50} + UV SFV. In the experiment shown in Table 12, 10% (1/10) of mice survived infection with 10 LD_{50} + UV SFV while 70% (7/10) of mice treated with DI SFV + 10 LD_{50} survived. To calculate the extent of protection, the percentage of mice surviving 10 LD_{50} + UV SFV is substracted from the percentage surviving after treatment with 10 LD_{50} + DI SFV. Thus in Table 12, the DI SFV protected 60% of mice. All future experiments will quote the protection rather than the actual number of survivors. With 10 LD_{50} + UV SFV there were either no survivors or 10% survivors. Non-inoculated control mice or control mice inoculated with UV SFV only or DI SFV only, survived infection. In all experiments control mice survived infection and will not be discussed further.

c) DI SFV preparations

Details of the passage history, haemagglutination assay, infectivity and interference titre (by both RSIA and YRA) of the DI SFV preparations used in protection experiments are shown in Table 13. As can be seen, in addition to DI virus DI SFV preparations contain between 10^5 and 10^8 pfu/ml S virus. Since in CFLP mice there are about 600 pfu/LD_{50} (in 20µl inoculated by the i.n. route) it was necessary to remove the excess infectivity present in DI virus preparations. Although it would be best to physically separate S and DI virus, this is not possible for alphaviruses (see General Introduction). An effective alternative procedure is to inactivate the larger S virus genome (4.3×10^6) by UV irradiation which will result in comparatively little inactivation of

Table 12 Protection of mice by i.n. inoculation of 10 LD_{50} and DI virus p13a

Inoculum	Survivors†		Protection†
	No.surviving / No.inoculated	%	
10 LD_{50}	1/10	10	
10 LD_{50} + UV SFV‡	1/10	10	
10 LD_{50} + DI SFV$	7/10	70	60
UV SFV only	4/4	100	
DI SFV only	4/4	100	
Uninfected*	4/4	100	

† Survivors at day 9 p.i.

‡ Protection is given by the proportion of mice protected from 10 LD_{50} by inoculation of DI SFV (70% here) minus the survivors of inoculation of 10 LD_{50} + UV SFV (10% here).

‡ UV SFV is UV irradiated non-infectious S virus diluted to a concentration of 4 HAU/ml.

$ DI SFV was UV irradiated to remove infectious virus so that no infectivity is detectable by plaque assay after UV irradiation.

* Control mice were mock-infected with diluent.

Table 13 Properties of DI SFV preparations used to inoculate mice

DI SFV PREPARATION				INTERFERENCE TITRE (DIU/ml)			
				RSIA		YRA	
Passage history	Antigenic mass (HAU/ml)	Infectivity (pfu/ml)	U.V.†	Before U.V.	After U.V.	Before U.V.	After U.V.
p4 (B4)*	4	1×10^8	100	56	8	126	⩽4
p5 (B5)	4	3×10^6	40	32	14	45	⩽4
p6 (B6)	⩽4	1×10^7	100	316	⩽4	501	⩽4
p8 (B8)	⩽4	1×10^6	60	178	100	447	N.D.
p9b (B9)	⩽4	2×10^8	50	20	8	20	⩽4
p9 (B8L1)	⩽4	1×10^8	40	20	11	18	⩽4
p11 (B11)	⩽4	2×10^6	60	32	⩽4	158	⩽4
p12g (B12)	⩽4	4×10^7	40	10	⩽4	71	⩽4
p13a (B13)	4	3×10^7	60	50	6	112	8
p13d (B13)	⩽4	3×10^5	40	80	16	126	8
p13h (B13)	4	1×10^6	45	63	28	200	12
p23 (B2IC2)	⩽4	2×10^6	60	20	⩽4	89	⩽4

* The designation indicates that this DI virus preparation has had four undiluted passages (p) all in BHK (B) cells. Lower case letters indicate sister stocks. Passage in L cells is indicated by L and in chick embryo cells by C.

† Time of U.V. irradiation(s).

the smaller DI virus genome ($\ll 1 \times 10^6$). The length of exposure was chosen to ensure that irradiated DI virus preparations contained no infectivity detectable by plaque assay. The time of UV irradiation used for each reference to DI virus preparation is shown in Table 13.

All reference to DI virus in this section is to UV-irradiated preparations. The effect of irradiation on interfering activity *in vitro* was assayed by the RSIA and YRA. Table 13 shows that there was significant inactivation of the interfering activity, but later results (see below) indicated that interference measured *in vitro* was of little relevance to the *in vivo* situation. Table 13 also shows that interference measured by RSIA was more resistant to UV inactivation than that recorded by YRA. The same was observed in the previous chapter (Figure 9). There was some variation in the rate of inactivation of interference measured by RSIA (for example, DI virus p6 was several times more sensitive than other DI virus preparations) which would imply that there are variations in the size of the DI virus genome(s).

d) Prevention of death in SFV-infected mice by DI SFV

The disease induced in adult CFLP mice inoculated i.n. with 10 LD_{50} S SFV follows a reproducible pattern (Table 14). Mice remain apparently healthy until 3 to 4 days p.i. when they show signs of malaise indicated by ruffled fur and inactivity. The first signs of central nervous system involvement are behavioural changes. These include "vertical walking" against the side of their cage and "continuous aimless walking" when they will walk over, rather than around, litter mates. Such

the smaller DI virus genome ($\ll 1 \times 10^6$). The length of exposure was chosen to ensure that irradiated DI virus preparations contained no infectivity detectable by plaque assay. The time of UV irradiation used for each reference to DI virus preparation is shown in Table 13.

All reference to DI virus in this section is to UV-irradiated preparations. The effect of irradiation on interfering activity *in vitro* was assayed by the RSIA and YRA. Table 13 shows that there was significant inactivation of the interfering activity, but later results (see below) indicated that interference measured *in vitro* was of little relevance to the *in vivo* situation. Table 13 also shows that interference measured by RSIA was more resistant to UV inactivation than that recorded by YRA. The same was observed in the previous chapter (Figure 9). There was some variation in the rate of inactivation of interference measured by RSIA (for example, DI virus p6 was several times more sensitive than other DI virus preparations) which would imply that there are variations in the size of the DI virus genome(s).

d) Prevention of death in SFV-infected mice by DI SFV

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Table 14 Clinical observations of DI SFV treated mice inoculated by i.n. route

Inoculum	Clinical symptom	Day post infection 0.8	1.7	2.7	3.7	4.7	5.7	6.8	7.8	survivors (%)	Protection (%)	Mean day of death ø
10 LD_{50} + UV SFV*	Dead‡					5	9	10				
	complete limb paralysis					4						
	hind limb paralysis											
	malaise				5		1					
	well	10	10	10	5	1				0		4.9
10 LD_{50} + DI SFV†	Dead‡					1		3				
	complete limb paralysis											
	hind limb paralysis											
	malaise				1		2					
	well	10	10	10	9	9	7	7	7	70	70	5.4

* UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml.

† DI SFV p13a.

‡ cumulative total

ø mean day of death is the sum of the day on which each mouse died divided by the number of dead mice.

alternations in the pattern of normal behaviour vary between individual mice. Malaise is succeeded by partial paralysis, usually of the hind limbs and this is followed by complete paralysis and death by day 5 or 6. Any survivors on day 7 survive the infection. The virology confirms that the brain is the target organ (see Figure 17), and that infectivity reaches titre of 10^9 to 10^{10} pfu/g tissue. Crouch et al. (1982) showed that there was a characteristic though not extensive histopathology, involving selective destruction of hippocampal pyramidal cells and perivascular cellular infiltration.

Table 14 shows the course of disease in DI virus-treated mice which either follow the disease pattern of mice treated with 10 LD_{50} + UV SFV or remain healthy throughout the whole experiment. Similarly there is no significant delay in death of DI virus-treated mice.

The reproducibility of the protection experiments is shown in Table 15. The mean percentage protection by DI SFV p13a is 58.6%. As can be seen the results are reproducible and, calculation of the 95% confidence limits (see Appendix) shows that there is little variation in the results (50.6% to 66.4%). Another DI virus, p4, which has also been used in many experiments (Table 15) gave a similar percentage (63.6%) protection and shows the reproducibility of protection between DI virus preparations.

Table 16 summarises the effects of different DI SFV preparations on the outcome of the lethal SFV encephalitis described above. All CFLP mice inoculated with S SFV together with all DI SFV preparations used in this

Table 15 Protection rate of mice treated with 10 LD_{50} + DI virus p4 or p13a

	Protection (%)	
	10 LD_{50} + DI SFV p4	10 LD_{50} + DI SFV p13a
Range	30, 46, 50, 57, 60, 60 67, 67, 70, 70, 80, 80, 90	29, 30, 40, 50, 50, 60, 60 62.5, 67, 70, 70, 70, 70, 70, 80
Mean	63.6 ± 4.4†	58.6 ± 4.0
95% confidence limits‡	55.0 - 72.2	50.8 - 66.4

† One standard error of the mean (S.E.M.)

‡ 95% confidence limits are ± 1.96 x S.E.M.

Table 16 Protection of mice against a lethal SFV encephalitis by administration of various preparations of DI SFV

DI SFV	Survivors♣	Mean (± S.E.M.)	Interference titre†	
Preparation	%	%	RSIA	YRA
p4	*	63.6 ± 4.4	8	⩽4
p5	0, 10	5.0	14	<4
p6	0, 0, 0	0.0 ± 0.0	⩽4	⩽4
p8	60, 70	65.0	100	N.D.
p9b	60, 67	63.5	8	⩽4
p91	30	-	11	⩽4
p11	30, 30	30.0	⩽4	⩽4
p12g	0, 0, 10	3.3 ± 3.3	⩽4	⩽4
p13a	*	58.6 ± 4.0	6	8
p13d	44, 50, 50, 70	53.5 ± 5.7	16	8
p13h	30, 50, 50, 60, 70	52.0 ± 6.6	28	12
p23	0, 0	0.0	⩽4	⩽4

† From Table 13; after UV-irradiation.

♣ At 9 days after p.i.; each figure represents a separate experiment in which 9 or more mice were inoculated with 10 LD_{50} • DI SFV.

* See Table 15.

study either follow the normal course of infection or show no sign of infection whatsoever. There are three categories of DI virus: 1. those which protect over 50% of infected mice given 10 LD_{50} (DI virus preparations p4, p8, p9b, p13a, p13d, p13h); 2. those which protect a minority of mice (p9l, p11, p12g), and 3. those which do not protect (p5, p6, p23). This variation was specific for individual DI virus preparations and the extent to which an infection was modulated was reproducible. DI virus p8 was the same preparation used by Dimmock and Kennedy (1978) and gave the same level of protection as described by these workers.

e) Correlation between protection by DI SFV and its passage history

No correlation was seen between the ability of DI viruses to protect mice and their passage history. For example, DI virus p4 gave protection but lost this property when passaged to produce DI virus p5 or p6. On the other hand, mouse protection can be gained on passage as DI virus p12, which protected poorly, gave rise to DI viruses of p13 which all gave good protection. However, the properties of DI virus preparations did not arise at random but were determined by the parental inoculum since the sister stocks p13a, p13d and p13h all had a similar ability to protect. Similar characteristics on passage were also seen with interfering properties in vitro (see Table 6).

f) Comparison between protection in vivo and interference titre-in vitro by DI SFV preparations

There was no correlation between ability of DI virus to protect mice and their interference titres in cell culture measured by the RSIA and YRA (Table 16). Although DI virus p8 had a high titre by RSIA it protected mice only as well as DI viruses p13a, p13d or p13h which had a 4-fold or more lower titre. DI viruses p5, p13a, p13d and p13h had similar interference titres by RSIA but only DI virus of the passage p13 level protected mice. Interference measured by the YRA was no better guide to the ability to protect mice since DI virus p4 and p9b, which had no detectable YRA titre, protected as well as DI viruses p13a, p13d and p13h which registered a positive value. It is concluded that DI virus which protects mice is qualitatively different from the DI virus which interferes in the in vitro assays.

g) Challenge of DI SFV-protected mice by S SFV at 21 days after the initial infection

Mice which had been protected by DI virus from a lethal SFV infection were challenged at 21 days p.i. with 100 LD_{50} i.n. to determine if they had developed a protective immune response (Table 17). All mice inoculated with UV SFV, the immunogen control which contained 4 HAU/ml,. succumbed to the challenge as did mice inoculated with the DI virus preparations alone. Thus the non-infectious virus preparations are not immunogenic.

When mice which had survived inoculation with 10 LD_{50} through

Table 17 Ability of mice which had been protected from SFV by administration of DI virus to survive a second inoculation of SFV†

First inoculum			Survivors	
10 LD_{50}	DI virus	UV SFV antigen	No. Surviving / No. Inoculated	%
-	-	-	0/19	0
-	-	+	0/17	0
-	p4	-	0/12	0
+	p4	-	1/21	5
-	p8	-	0/8	0
+	p8	-	1/18	6
-	p9b	-	0/6	0
+	p9b	-	6/12	50
-	p11	-	0/8	0
+	p11	-	1/8	13
-	p13a	-	2/11	18
+	p13a	-	17/21	81
-	p13h	-	0/8	0
+	p13h	-	11/13	85

† Mice were given 100 LD_{50} i.n. 21-24 days after the first inoculum. Results shown are with mice taken from the type of experiment described in Table 16.

Table 18 Ability of mice which had survived administration of 10 LD_{50} + UV DI virus, or DI virus only to survive a second inoculation of 10 LD_{50} S SFV

First inoculum	Challenge survivors* No. surviving / No. inoculated	%
Uninfected†	0/9	0
UV DI virus p4	0/4	0
10 LD_{50}+UV DI virus p4	14/15	93
UV DI virus p13a	0/4	0
10 LD_{50} + UV DI virus p13a	3/4	75

* Mice were given 10 LD_{50} i.n. 21-24 days after the first set of inoculations.

† Mice were inoculated with diluent only.

administration of DI viruses p6, p9b, p13a or p13h were challenged 21 days later with 100 LD_{50} SFV, the majority (>50%) were completely resistant and showed no signs of infection. However, those mice which had received 10 LD_{50} plus DI viruses p4, p8 or p11 were susceptible to the challenge and died after infection had followed its normal course.

Thus although DI viruses p4 and p13a modulated the primary SFV infection to the same extent (64 and 59% survivors respectively, Table 15) they differed entirely in their ability to establish an adaptive immunity against the challenge by 100 LD_{50} S virus. To determine whether adaptive immunity was all-or-nothing mice protected by DI viruses p4 and p13a were challenged with 10 LD_{50} S virus at 21 days p.i. (Table 18). The results show that DI virus p4 treated mice are resistant to challenge by 10 LD_{50} S virus while control mice succumbed. Thus the extent of adaptive immunity of mice infected with different DI virus preparations varies rather than being all-or-nothing. This may explain the result of mice treated with 10 LD_{50} + DI SFV p9b where 50% survive challenge with 100 LD_{50} (Table 17).

h) <u>DI virus preparations standardised for haemagglutination and interference titre</u>

For clarity, the different types of modulatory effects of DI SFV preparations described above are illustrated by reference to 3 DI virus preparations, p4, p5 and p13a (Table 19). These 3 DI viruses contain the same amount of SFV antigen by haemagglutination (4 HAU/ml) and similar interference titres by RSIA. DI viruses p4 and p5 had no

Table 19 Comparison of properties of DI SFV preparations

DI Virus preparation	Antigenic mass (HAU/ml)†	Interference titre‡ (DIU/ml) RSIA	YRA	Protection‡ (%)	Challenge survivors (%)‡
p4	4	8	≤4	64	5
p5	4	14	≤4	5	N.A.
P13a	4	6	8	59	81

† Data taken from Table 13

‡ Data taken from Table 16

‡ Data taken from Table 17

N.A. = Not applicable

detectable interference titre by YRA, while DI virus p13a had a low titre (8 DIU/ml). DI viruses p4 and p13a protected similar numbers of mice (64 and 59% respectively) while DI virus p5 protected only 5% (Table 16). Although DI viruses p4 and p13a protected similar numbers, DI virus p4-treated mice were susceptible to challenge by 100 LD_{50} S SFV, while p13a-treated mice were resistant. Thus, although all 3 DI virus preparations contained the same amount of SFV antigen and similar interference titres as measured by RSIA there were clearly two categories of DI virus, those which protected mice (p4 and p13a) and those which did not (p5). Similarly although different DI virus preparations may protect similar numbers of mice, the mice may have weak or strong adaptive immune responses. It is concluded that there are at least 3 qualitatively different categories of DI virus preparations with respect to their ability to protect mice against a lethal SFV encephalitis.

i) Neutralizing antibody induced during SFV infections of mice

The inability of some DI SFV preparations to protect against re-infection by 100 LD_{50} SFV was further investigated by measuring serum and brain neutralizing antibody (Table 20). As a control, mice were inoculated i.n. with avirulent SFV and these mice all responded with a mean serum titre of 1683 (Table 20). In contrast, both groups of mice protected by DI virus responded poorly and few p4 (3/11) and p13a (3/12) treated mice had titres exceeding 10% of the avirulent value. These results indicate that the ability of DI SFV p13a to initiate a strong immune response is not reflected by the serum neutralizing antibody

Table 20 Brain and serum neutralizing antibody titres in individual CFLP mice which had survived inoculation of 10 LD_{50} SFV + DI virus, or had received avirulent SFV, intranasally

Inoculum	Sample	Titre of neutralizing antibody[+]
mock-infected	brain	⩽9*, ⩽9, ⩽9
	serum	⩽2, ⩽2, ⩽2
avirulent†	brain	⩽9, 16, 35, 89, 158
	serum	3162, 1778, 891, 1584, 1000
DI virus p4 only	brain	⩽9, ⩽9, ⩽9, N.D.
	serum	⩽2, ⩽2, ⩽2, ⩽5
10 LD_{50} + DI virus p4	brain	⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, N.D.
	serum	⩽2, ⩽5, ⩽9, ⩽9, ⩽20, ⩽2, 9, 32, 2000, 2000, 3162
DI virus p13a only	brain	⩽9, 9, 9, N.D.
	serum	⩽2, ⩽2, ⩽5, ⩽5
10 LD_{50} + DI virus p13a	brain	⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, ⩽9, 14, 14, N.D.
	serum	⩽9, ⩽9, ⩽9, 9, 10, 18, 22, 398, 1000, 141, 501, 22

[+] At day 13/14 post infection. Titre is the reciprocal of the dilution giving 50% plaque reduction.

† Mice were inoculated intranasally with 6×10^4 pfu of avirulent SFV.

* Antibody titres from the brain and serum of the same mouse are shown together in vertical pairs. Each brain was suspended in 4 ml medium 199 + 2% calf serum. There is approximately 4 ml serum in CFLP mice (Kaliss and Pressman, 1950). Therefore, brain and serum antibody titres are directly comparable.

N.D. = not done

titres. By comparison, all brain neutralizing antibody titres were low or undetectable: avirulent SFV-infected mice had low titres (average = 61), while of mice inoculated with 10 LD_{50} virulent SFV only 2/11 treated with DI virus p13a had detectable antibody (titre = 14).

For DI virus-treated mice there was little correlation between the titre of neutralizing antibody in the serum and that in brain. IgM antibody levels were not determined.

j) Effect of DI SFV upon a heterologous virus infection

Since DI viruses p4 and p13a protect similar numbers of mice against 10 LD_{50} yet differ in their post-infection immune status, it is possible that DI SFV p4 protected mice by stimulation of a non-adaptive immune response such as interferons, natural killer cells or macrophages. This was examined by co-inoculating mice with a heterologous neurotropic picornavirus, encephalomyocarditis (EMC) virus. EMC virus causes a flacid paralysis readily distinguishable from SFV and death ensues at 4 days p.i. Mice inoculated with DI SFV p4 or p13a + 10 LD_{50} SFV + 10 LD_{50} EMC all died although in parallel groups the DI viruses protected against SFV alone as expected (Table 21). Calculation of the mean day of death showed that DI SFV did not delay death in mice inoculated with EMC. These findings suggest that there is no overwhelming non-adaptive immune response involved in the protection of mice against 10 LD_{50} SFV by either DI SFV p4 or p13a.

Table 21 Effect of DI SFV on a heterologous virus infection in mice*

Inoculum+	Survivors†	%	Mean day of death‡
EMC	0/10	0	4.1
EMC + UV SFV	0/8	0	4.1
SFV + UV SFV	0/10	0	4.9
SFV + EMC + UV SFV	0/10	0	4.9
DI virus p13a + SFV	7/10	70	5.4
DI virus p13a + EMC	1/10	10	3.7
DI virus p13a + EMC + SFV	0/11	0	4.6
DI virus p4 + SFV	5/10	50	5.2
DI virus p13a + EMC	0/10	0	4.6
DI virus p13a + EMC + SFV	0/11	0	5.1

* Mice were inoculated according to Methods. For EMC in CFLP mice there are 100 pfu/LD_{50} (in 20 µl inoculated by the i.n. route).

+ 10 LD_{50} EMC; 10 LD_{50} SFV.

† number of mice surviving/ number of mice inoculated.

‡ Mean day of death is the sum of the day on which each mouse died divided by the number of dead mice.

k) Do interferons affect protection of mice by DI SFV?

The results described above with EMC virus suggest that non-adaptive immune responses such as interferon are not involved in protection. To examine this possibility further, the DI virus preparations used in protection experiments were assayed for the presence of interferon. Since DI SFV preparations are propagated in BHK cells and used in mice they were tested for the presence of hamster or mouse α and β interferons. Table 22 shows that no interferons were detected in any of the DI SFV preparations assayed (p4, p12g, p13a) and that hamster interferon was inactive on mouse cells and vice versa.

A different way to test for the possible role of interferon was to actually add the substance to DI virus prior to co-inoculation of mice. Table 23 shows that 1000 units of $\alpha\beta$ intereron (in a 20 µl volume) did not prevent the disease caused by 10 LD_{50} S SFV. Also this dose of interferon did not enhance protection by DI viruses p4 and p13a, and the non-protecting DI SFV p12g did not protect when interferon was added to the inoculum. Therefore there is no evidence to suggest that interferon plays any role in the protection of mice by DI SFV preparations.

l) Effect of mouse-strain upon the extent of protection by DI SFV

Different mouse strains were compared to see if the genetic background of the host influenced their capacity to be protected by DI virus against the lethal encephalitis caused by i.n. inoculation of 10 LD_{50} S virus (Table 24). Both CFP and Porton random bred strains of mice had a similar pfu:LD_{50} ratio (6×10^2 pfu) while that of an inbred strain,

Table 22 Presence of Interferon in DI SFV preparations

Sample†	Interferon Titre† (log_{10} units/ml)	
	Mouse Cells	Hamster Cells
DI SFV p4	≤0.6	≤0.6
DI SFV p12g	≤0.6	≤0.6
DI SFV p13a	≤0.6	≤0.6
Mouse interferon	3.2	≤1.6
Hamster interferon	≤1.6	3.0

† DI virus preparations were treated as described in Methods to remove biologically active virus.

† Samples were titrated on L_{929} cells to measure mouse interferon and Chinese hamster ovary cells to measure hamster interferon. interferon standards were prepared by induction of the two cell types above with Newcastle disease virus. Therefore these standards consist of a mixture of α and β interferon species. Interferon titres are expressed in arbitary units.

Table 23 Protection of mice by 10 LD_{50} + DI SFV with the addition of interferon to the inoculum

Inoculum†	Protection (%)
10 LD_{50} + UV SFV	0
" + UV SFV + IFN*	10
" + DI SFV p4	73
" + DI SFV p4 + IFN	60
" + DI SFV p12g	0
" + DI SFV p12g + IFN	0
" + DI SFV p13a	55
" + DI SFV p13a + IFN	50

† Mice were inoculated with the protocol described in the Methods. Where indicated 10^3 units of IFN was present in each inoculum.

* IFN = interferon.

Table 24 Variation in the ability of different strains of mice to be protected from SFV-mediated lethal encephalitis by administration of DI SFV

Mouse strain	PFU/LD_{50}	Protection† (%)	
		$10LD_{50}$ DI virus p8	$10LD_{50}$ DI virus p13a
Porton	6×10^2	12.5	N.D.
CFLP	6×10^2	70	70
C_3H-He/Mg	1.5×10^4	N.D.	0

† Protection is number surviving/number inoculated

N.D. = not done

C3H-He/Mg was 1.5 x 10^4 pfu. Inoculation of DI SFV p8 plus 10 LD_{50} into CFLP mice results in 70% of mice surviving the lethal encephalitis, while inoculation of the same virus combination into Porton mice gave only 12.5% survivors. Since the LD_{50} of S virus is the same in both strains of mice, it is concluded that host factors are influencing the extent of DI virus-mediated protection. Inoculation of DI SFV p13a plus 10 LD_{50} into CFLP and C3H-He/Mg mice protected 70% of CFLP mice but none of the C3H-He/Mg treated mice. As with experiments with CFLP mice, Porton and C3H-He/Mg mice inoculated with DI SFV plus 10 LD_{50} either remained completely healthy throughout the experiment or died of apparently the same disease contracted by mice inoculated with S SFV alone. Death was not delayed nor were there any aberrant signs of disease in DI SFV-treated mice. The lack of protection in C3H-He/Mg mice probably results from the 25-fold greater amount of S SFV required for a lethal dose.

Although these results indicate that host factors are involved in the protection phenomenon, Table 25 shows that protection of CFLP mice was not affected by the sex of mice inoculated. DI viruses p4 and p13a protected both sexes equally.

m) Effect of concentration of DI and S virus upon protection by DI SFV

The amount of DI virus required for protection was determined by inoculating mice with serial dilutions of DI SFV together with 10 LD_{50} S SFV (Table 26). The results show that with both DI viruses examined (p4 and p13a) the concentration of DI virus inoculated was critical for

Table 25 Protection of male and female CFLP mice from SFV-mediated encephalitis by the administration of DI virus

Inoculum	Protection† (%)	
	Male mice	Female mice
10 LD_{50} + DI virus p4	77	64
10 LD_{50} + DI virus p13a	45	41

† Protection is number surviving/number inoculated

At least 14 mice were inoculated in each group.

Table 26 Protection of CFLP mice against 10 LD_{50} S SFV using dilutions of DI SFV

Dilution of DI virus	Protection† (%)	
	10 LD_{50} DI virus p4	10 LD_{50} DI virus p13a
1/1	73	60
1/2	9	20
1/5	10	10
1/10	0	0

† Protection is number surviving/number inoculated.

protection of mice, since a 2-fold dilution of DI virus was sufficient to reduce protection. This result may explain why some DI SFV preparations appear unable to protect mice although there was still slight protection with a 5-fold dilution of DI virus.

To follow this up it was determined if protection depended upon the absolute amount of DI virus inoculated per mouse or the ratio of DI virus: S virus. Table 27 shows that increasing the quantity of S virus inoculated reduced protection by both DI virus p8 and p13a, suggesting that the ratio of DI virus to infectivity is a critical factor in protection.

n) Infectivity content of DI SFV preparations

As described above (section c) DI SFV preparations were UV irradiated until they contained no infectious virus detectable by plaque assay ($\leq$2.5 pfu/ml) before administration into mice. On a few occasions some mice inoculated with such apparently non-infectious preparation died with a typical SFV disease. To understand why these mice were dying two sets of experiments were performed. Firstly, three DI virus preparations were each UV irradiated for 50 sec to reduce the amount of infectivity present (Table 28). The plaque assays showed a lack of proportionality in the dose-response relationship since more plaques were obtained at 1/10 dilution than at undiluted.

Secondly, it was determined whether or not the DI virus present in a DI virus preparation could inhibit the formation of plaques by adding a

Table 27 Protection of mice by DI virus against inoculation of various amounts of S SFV

No. LD_{50} inoculated	Protection (%)	
	DI SFV p8†	DI SFV p13a†
3	25	N.D.
10	12.5	60
20	N.D.	30
30	0	13

† DI SFV p8 was inoculated into Porton mice and DI SFV p13a into CFLP mice.

N.D. = Not done

Table 27 Protection of mice by DI virus against inoculation of various amounts of S SFV

No. LD_{50} inoculated	Protection (%)	
	DI SFV p8†	DI SFV p13a†
3	25	N.D.
10	12.5	60
20	N.D.	30
30	0	13

† DI SFV p8 was inoculated into Porton mice and DI SFV p13a into CFLP mice.

N.D. = Not done

Table 28 Infectivity titration of UV irradiated DI SFV preparations

DI SFV preparation	dilution of plaque assay of UV irradiated DI SFV†		
	1/1	1/10	1/100
p5	0	1	0
p13d	3	9	1
p13h	0	4	0

† 1 ml samples of each DI virus preparation were taken and UV irradiated for 50 sec. according to the procedure described in Methods. Residual infectivity was titrated by plaque assay.

known number of pfu to UV-irradiated DI virus (Table 29). DI SFV preparation p13d which had been irradiated for 50 sec was used as a diluent for a preparation of S virus which itself contained no detectable DI virus by either RSIA or YRA.

The infectivity titration using a PBS diluent produces a normal plaque assay titration and DI virus alone gave a small number of plaques. However, with undiluted UV DI SFV as diluent, plaque formation was greatly inhibited. This inhibition diluted out at 1/10 and 1/100.

Thus the plaque assay is not a reliable guide to residual infectivity present in DI virus preparations and the presence of a considerable amount of infectious virus could be concealed. To overcome this problem all UV DI SFV preparations were tested in mice prior to use in protection experiments. The above findings may explain the different results obtained for the ratio of biologically active DI virus to particle quoted in the literature (Schlesinger et al., 1972; Logan, 1979; Kowal and Stollar, 1980).

Discussion

Evidence has been presented that DI SFV preparations are heterogeneous in their ability to protect mice. Some protect and others do not; of those which protect some (e.g. DI SFV p13a) confer strong immunity to a subsequent lethal challenge, whilst others (e.g. DI SFV p4) leave the mice with weak immunity. It would appear that the extent of immunity to

Table 29 Infectivity titration of a S virus preparation using a DI SFV preparation as diluent

Log_{10} dilution of DI SFV used as diluent	Log_{10} dilution of S virus in DI SFV "diluent"†					
	5	6	7	8	9	None
0	30	15	10	5	0	3
1	CON†	CON	53	22	11	9
2	CON	CON	35	4	5	1
3	CON	CON	28	2	0	N.D.♣
PBS♦	CON	CON	37	3	0	

† DI SFV preparation pl3d was UV irradiated for 50 sec and then used as diluent for an infectivity titration of a S virus preparation.

♦ PBS was used as diluent in place of UV DI SFV.

♣ CON: confluent plaques; N.D.: not done.

Table 29 Infectivity titration of a S virus preparation using a DI SFV preparation as diluent

Log_{10} dilution of DI SFV used as diluent	Log_{10} dilution of S virus in DI SFV "diluent"†					
	5	6	7	8	9	None
0	30	15	10	5	0	3
1	CON†	CON	53	22	11	9
2	CON	CON	35	4	5	1
3	CON	CON	28	2	0	N.D.♣
PBS♦	CON	CON	37	3	0	

† DI SFV preparation p13d was UV irradiated for 50 sec and then used as diluent for an infectivity titration of a S virus preparation.

♦ PBS was used as diluent in place of UV DI SFV.

♣ CON: confluent plaques; N.D.: not done.

challenge varies with the DI virus used to protect mice. Protection varied independently of passage history but sister stocks protected mice to similar extents. This variability of DI SFV *in vivo* is consistent with the findings that interfering properties of DI SFV *in vitro* are heterogeneous (see Results, Chapter 1). None of the 3 aspects of interference studied *in vitro* (RSIA, YRA and virus protein synthesis) appear to correlate with interference in the infected mouse. Thus the RSIA and YRA cannot be used to predict which DI virus preparations modulate SFV infections *in vivo* and consequently may be of no use in measuring DI virus in the mouse (see Results, Chapter 3).

Protection was shown to result in mice being either susceptible or resistant to re-challenge with 100 LD_{50} depending upon the DI SFV preparation used. Although mice treated with 10 LD_{50} + DI virus p4 were susceptible to challenge with 100 LD_{50}, they resisted challenge with 10 LD_{50}. Clearly immunity to challenge is not all-or-nothing but rather strong or weak depending on the DI virus preparation used to protect mice. If DI viruses such as p4 only stimulate weak adaptive immune rsponses, it is difficult to envisage these processes contributing greatly to recovery from the SFV infection. With such SFV DI virus preparations other factors must be involved in protection since DI SFV preparations p4 (weak adaptive immune response) and p13a (strong adaptive immune response) protected similar numbers of mice (64 and 59% respectively). Possibly, protection is mediated entirely through DI virus-mediated interference as with DI lymphocytic choriomeningitis virus (Welsh *et al.*, 1977), or DI virus stimulates non-adaptive immune responses (such as interferons, macrophages, natural killer cells) as

suggested for DI VSV in hamsters (Fultz et al., 1982a). The fact that the SFV disease is so rapid also suggests that adaptive responses will not play a role in recovery. Stimulation of non-adaptive responses was investigated by co-inoculation with EMC virus, which is sensitive to the effects of interferon (Gresser et al., 1976). No modulation of the EMC virus infection was observed by administration of DI SFV suggesting that non-adaptive immune responses are not involved in protection. However, it should be remembered that the above experiment cannot exclude a localized non-specific response to infection which would affect SFV but not EMC virus. The possibility that protection was mediated through interferon present in the inoculum was discounted (Table 22), and similarly it was shown that administration of interferon in the inoculum did not enhance protection. Although interferon has not been assayed in the DI virus-treated mice, the results above suggest that interferon does not play a role in the protection of mice by DI virus. The current situation argues that protection of mice by some DI virus preparations may be mediated through DI virus-mediated interference and/or non-specific immune responses yet to be identified.

Chapter 3

Inhibition of SFV multiplication in tissues of DI virus-treated mice

Introduction

The studies in the previous chapter have shown that DI SFV preparations can protect mice against 10 LD_{50} S SFV. Therefore, it was decided to investigate interference in virus multiplication in the mouse. DI virus p4 and p13a protected similar numbers of mice, but those protected by DI virus p4 had a weak adaptive immune response while those protected by DI virus p13a had a strong adaptive immune response. This suggests differences in the way in which these 2 DI viruses protect mice and therefore both viruses were further investigated. Mice were inoculated as before with 10 LD_{50} together with DI SFV p4 or p13a, and at intervals after infection, tissues were examined for infectivity and the presence of DI virus.

Results

a) Growth curve of infectivity in mice after i.n. inoculation

Figure 17 shows the growth curve of 10 LD_{50} (600 pfu/LD_{50}) S SFV in CFLP mice after i.n. inoculation. The serum, spleen, brain (minus olfactory lobes) and olfactory lobes were assayed for infectivity. Virus was first detected in the olfactory lobes at 12 h and this increased up to 3 days p.i. Virus appeared to spread posteriorly and appeared in the rest of the brain at 1 day p.i. Infectivity increased until 4 days p.i. when peak titres (up to 10^{10} pfu/brain) were detected. Virus was only detected in spleen and serum from day 2 p.i. where virus titres reached 10^{6} pfu/mouse. Mice showed clinical signs of disease on day 3

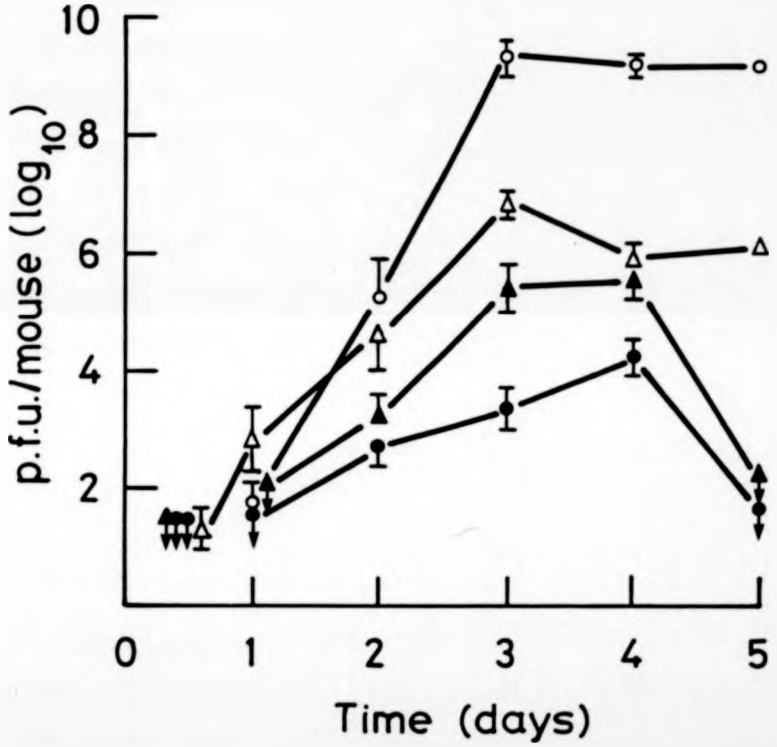

10
8
6
4
2
p.f.u./mouse (log10)
0
1
2
3
4
5
Time (days)

Figure 17. Growth curve of S SFV in CFLP mice after i.n. inoculation of 10 LD_{50} + UV SFV

Groups of 8 mice were killed at various times p.i. and tissues assayed for infectivity. Error bars are ± 1 S.E.M. On day 5 p.i. only one mouse was alive for sampling. brain (minus olfactory lobes) o; olfactory lobes △; spleen ●; serum ▲.

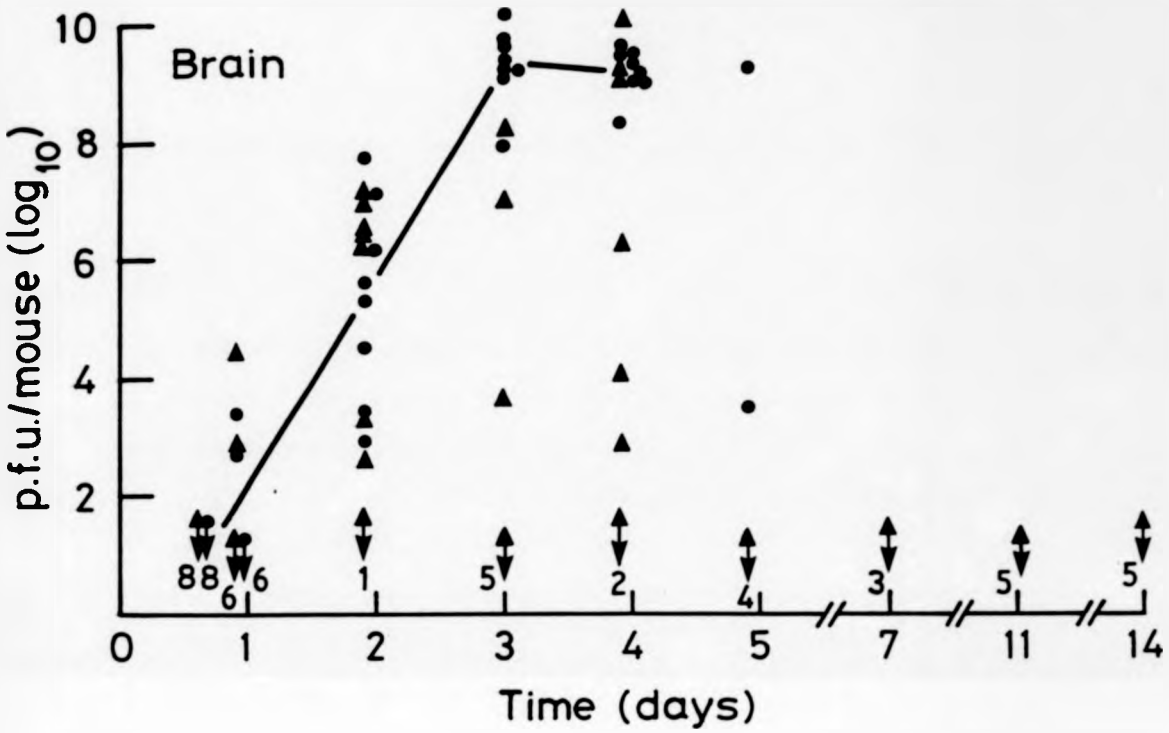
and died either on day 4 or day 5 p.i. It is rare for an animal to survive until day 6.

b) Multiplication of virus in mice co-infected with DI and S SFV

Groups of 8 mice were inoculated with DI SFV plus 10 LD_{50} S virus or 10 LD_{50} S virus plus UV SFV according to the protocol described previously (Dimmock and Kennedy, 1978). Mice were killed at intervals after inoculation and the infectivity present in serum, spleen, brain (less olfactory lobes) and olfactory lobes determined. The results obtained for DI SFV preparations p4 and p13a are shown in Figures 18 and 19 respectively. Death of mice after day 4 p.i. reduced numbers of mice available for sampling.

c) Mice treated with DI SFV p4

Comparison of the infectivity titres in mice treated with 10 LD_{50} + DI SFV p4 with those found in mice inoculated with 10 LD_{50} + UV SFV (Figure 18) shows that the DI virus-treated mice can be divided into two classes: 1) those which have virus titres reduced by $\geq$99% and, 2) those which have titres which were not significantly different. The distribution of the two classes of virus titres in the four tissues examined is shown in Table 30. Overall 58% of the DI virus treated mice infectivity titres from the four tissues examined were in the reduced infectivity group. This figure compares favourably with lethality studies where 64% survived (Table 15). Thus the first group can be equated with mice which would have been protected from death by DI virus


Brain
p.f.u./mouse (log10)
10
8
6
4
2
8 8 6 6
1
5
2
4
3
5
5
0
1
2
3
4
5
7
11
14
Time (days)

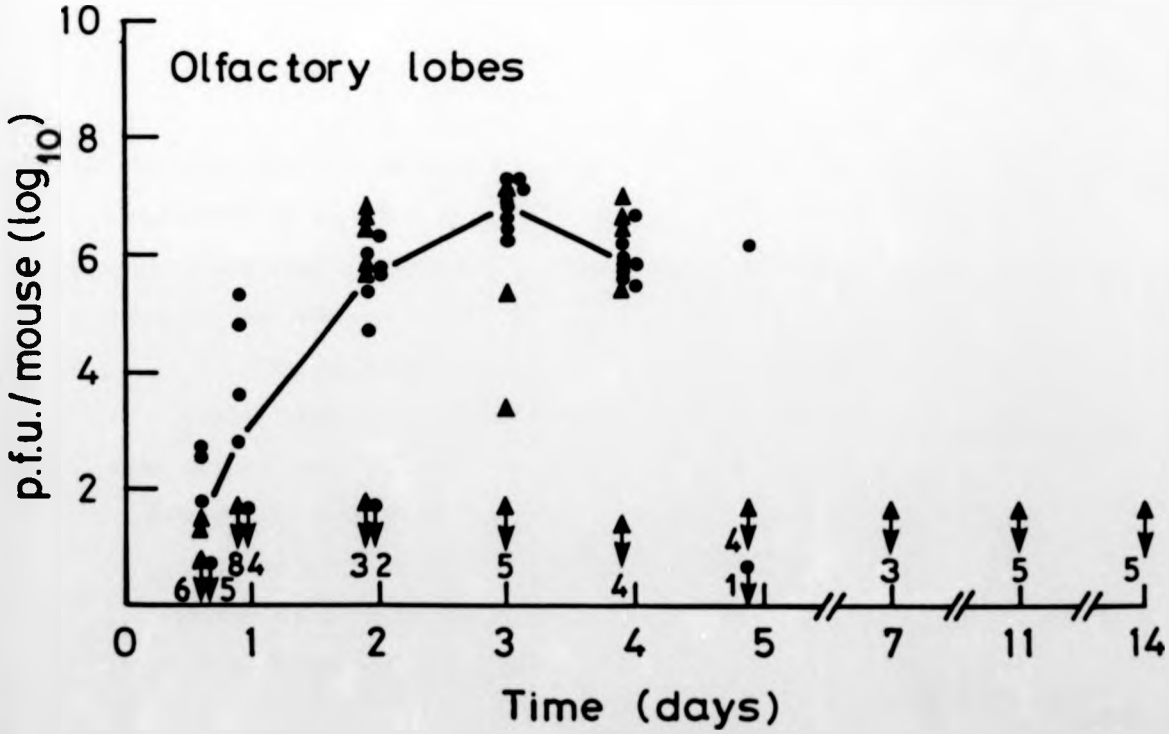
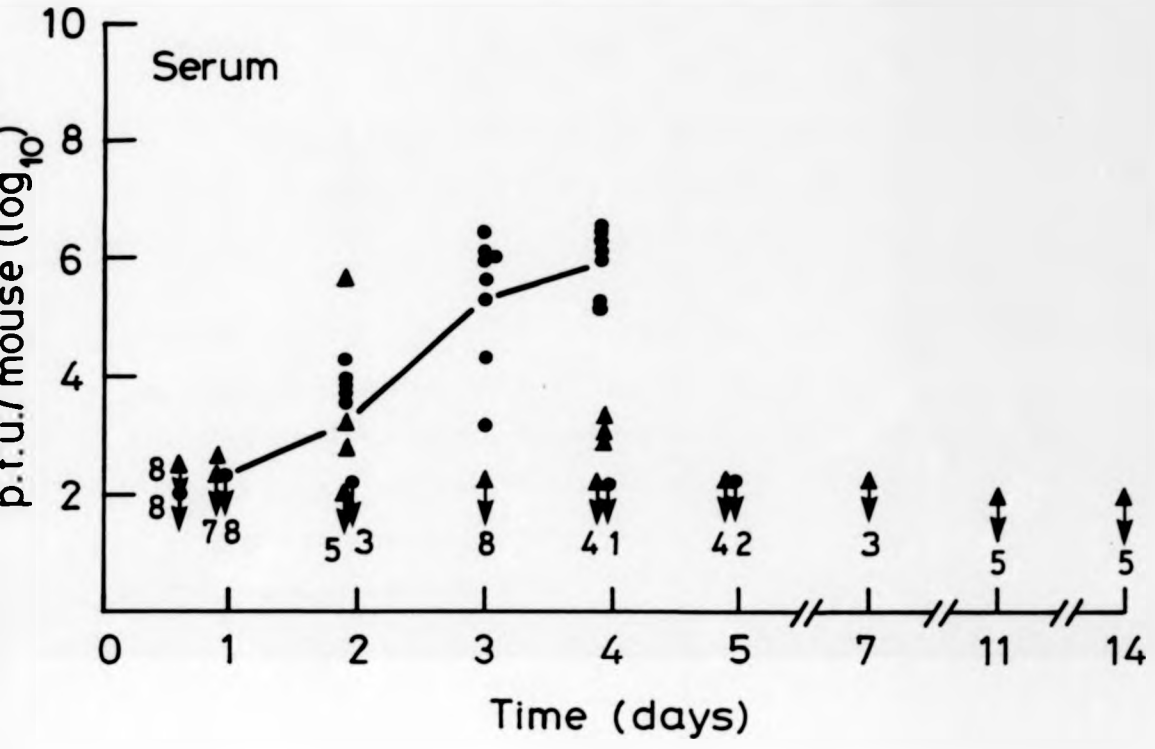

Olfactory lobes
p.f.u./mouse (log10)
10
8
6
4
2
6 5 8 4
3 2
5
4
4 1
3
5
5
0
1
2
3
4
5
7
11
14
Time (days)

Figure 18a Multiplication of SFV in mice inoculated with 10 LD_{50} and treated with DI SFV p4 - brain, and olfactory lobes

Mice were inoculated with 10 LD_{50} + UV SFV or 10 LD_{50} + DI SFV p4 and sacrificed at various times after infection. Curves represent mean infectivity titres of detectable virus for sample 10 LD_{50} + UV SFV. Arrows with adjacent number refers to the number of mice with undetectable infectivity. 10 LD_{50} + UV SFV ●; 10 LD_{50} + UV DI SFV p4 ▲.


Serum
p.f.u./mouse (log_{10})
10
8
6
4
2
8 8 7 8
5 3
8
4 1
4 2
3
5
5
0 1 2 3 4 5 7 11 14
Time (days)

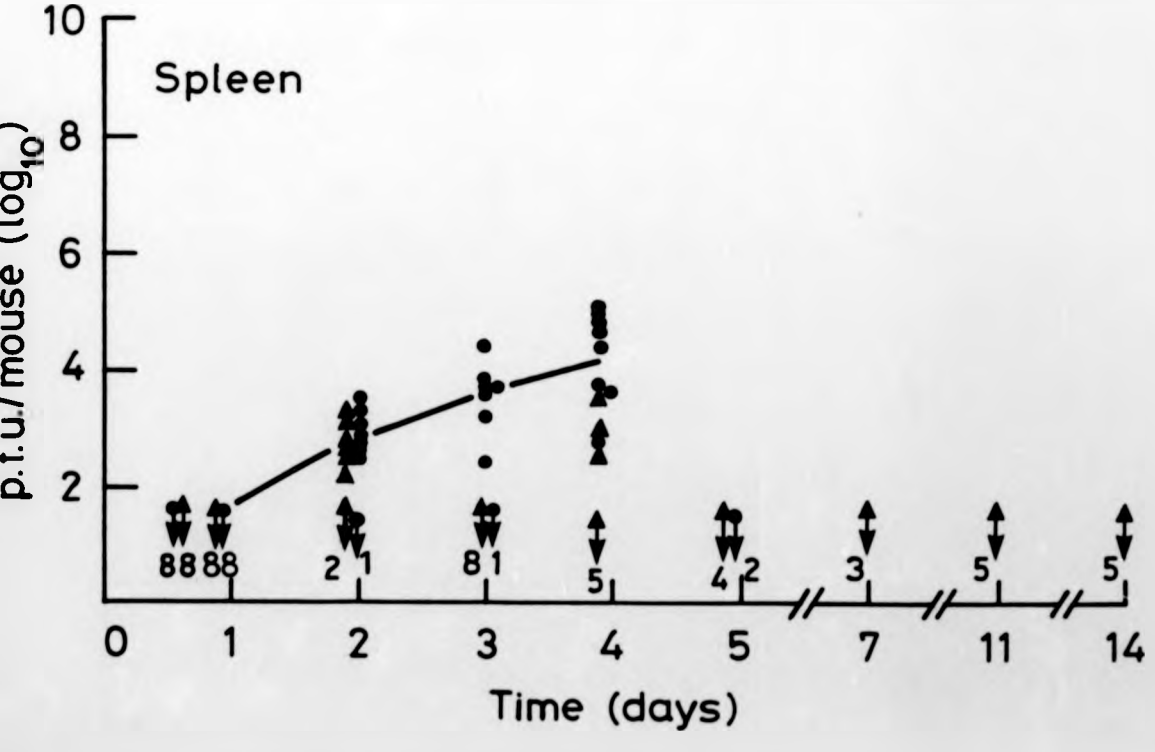

Spleen
p.f.u./mouse (log_{10})
10
8
6
4
2
8 8 8
2 1
8 1
5
4 2
3
5
5
0 1 2 3 4 5 7 11 14
Time (days)

Figure 18b Multiplication of SFV in mice inoculated with 10 LD_{50} and treated with DI SFV p4 - serum, and spleen

Mice were inoculated with 10 LD_{50} + UV SFV or 10 LD_{50} + DI SFV p4 and sacrificed at various times after infection. Curves represent mean infectivity titres of detectable virus for sample 10 LD_{50} + UV SFV. Arrows with adjacent number refers to the number of mice with undetectable infectivity. 10 LD_{50} + UV SFV ●; 10 LD_{50} + UV DI SFV p4 ▲.

Table 30 Proportion of infectivity in tissues from mice treated with 10 LD_{50} + DI SFV p4

Time p.i. (days)	Proportion of infectivity titres reduced in tissue sample†			
	olfactory lobes	brain	spleen	serum
0.5	0/8	0/8	0/8	0/8
1	8/8	0/8	0/8	0/8
2	3/8(-2)*	2/8	2/8(-1)	5/8(-3)
3	6/8	7/8	8/8(-1)	8/8
4	4/8	5/8	5/8	7/7(-1)
5	4/4	4/4	4/4(-2)	4/4(-2)

† No. mice with reduced infectivity/No. mice sampled.
Reduced infectivity is defined arbitrarily as >99% reduction in infectivity compared to mean infectivity of mice treated with 10 LD_{50} + UV SFV. All samples after day 5 p.i. had reduced infectivities.

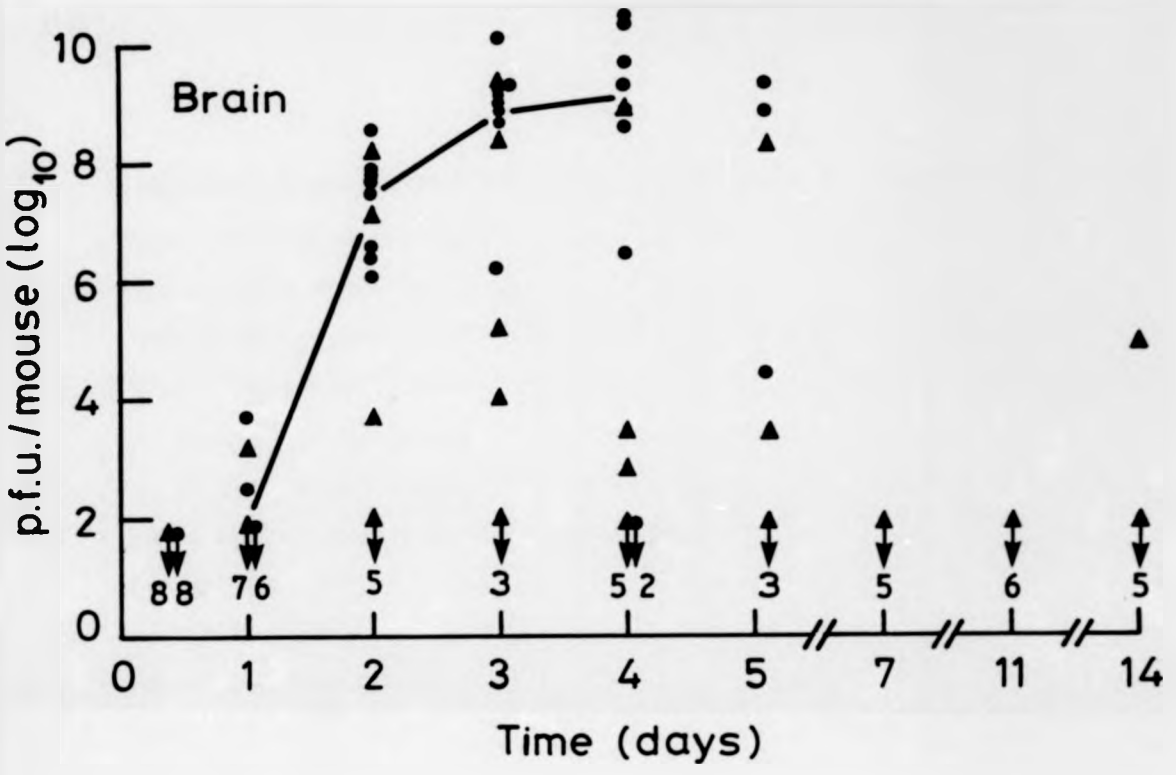
* number in bracket refers to number of mice from group infected with 10 LD_{50} + UV SFV which have "reduced" or no detectable infectivity.

Data from Fig. 18.

and the second group with mice which would have died with the disease following its normal course. Of course, interpretation would be easier had DI virus been 100% protective. Examination of Figure 18 shows that up to day 2 p.i. infectivity titres in the brain, olfactory lobes and spleen in DI virus-treated and non-treated mice were virtually identical, whereas infectivity titres in the serum were lower in DI virus-treated mice than non-treated mice. However, the majority of samples from DI virus-treated mice taken from day 3 onwards have lower infectivity titres than those of untreated mice. Many of these samples, and all taken from day 7 p.i. onwards, had undetectable levels of infectivity (brain 83%, olfactory lobes 96%, spleen 100%, serum 90% of mice sampled contained $\leqslant$400 pfu/tissue).

d) Mice treated with DI SFV p13a

Infectivity titres obtained from mice infected with 10 LD_{50} plus DI SFV p13a can again be divided into two classes (Figure 19). The distribution of the two classes of infectivity titres in the four tissues examined is shown in Table 31. Sixty-eight percent of all the infectivity titres from the four tissues examined were in the group defined as having titres reduced by $\geqslant$99% compared with mice not treated with DI virus. This figure is close to the 59% survival achieved by treatment with DI SFV p13a in lethality experiments (Table 15). The pattern of infectivity titres in DI SFV p13a-treated mice was different from that found in DI SFV p4-treated mice. Whereas the latter group had infectivity titres identical to non-treated mice up to day 2 p.i., mice which had been inoculated with 10 LD_{50} plus DI SFV p13a had reduced


Brain
p.f.u./mouse (log10)
10
8
6
4
2
0
8 8
7 6
5
3
5 2
3
5
6
5
0
1
2
3
4
5
7
11
14
Time (days)

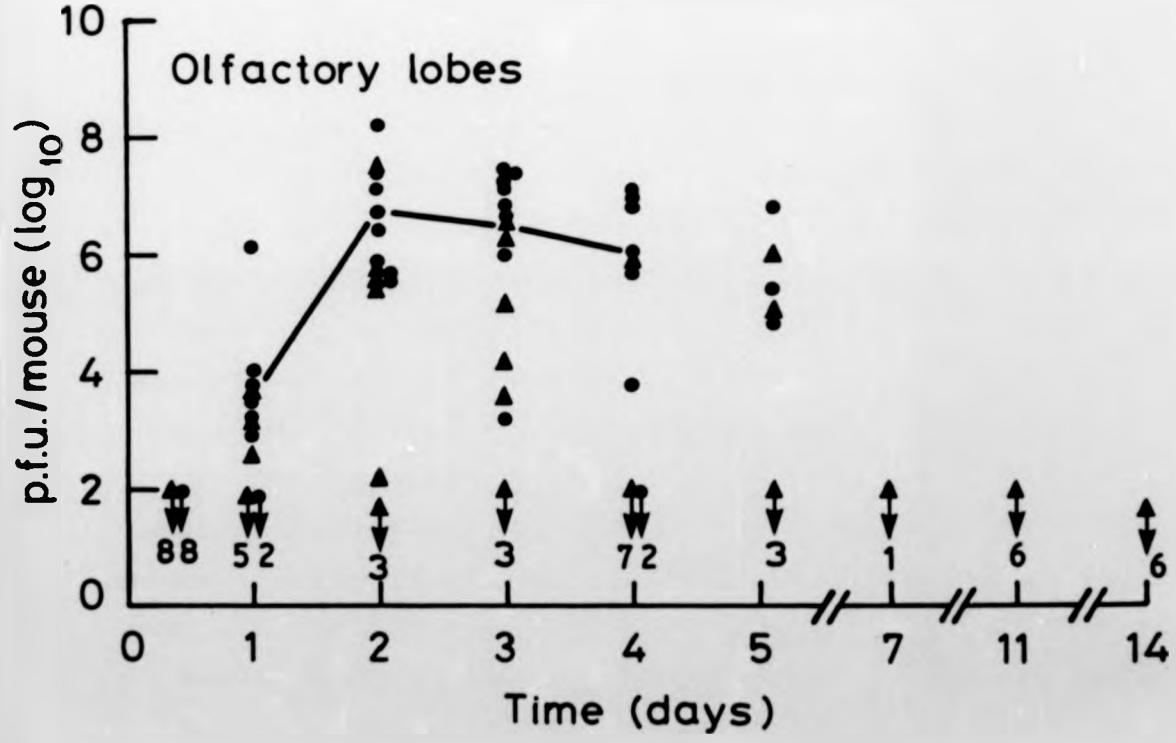
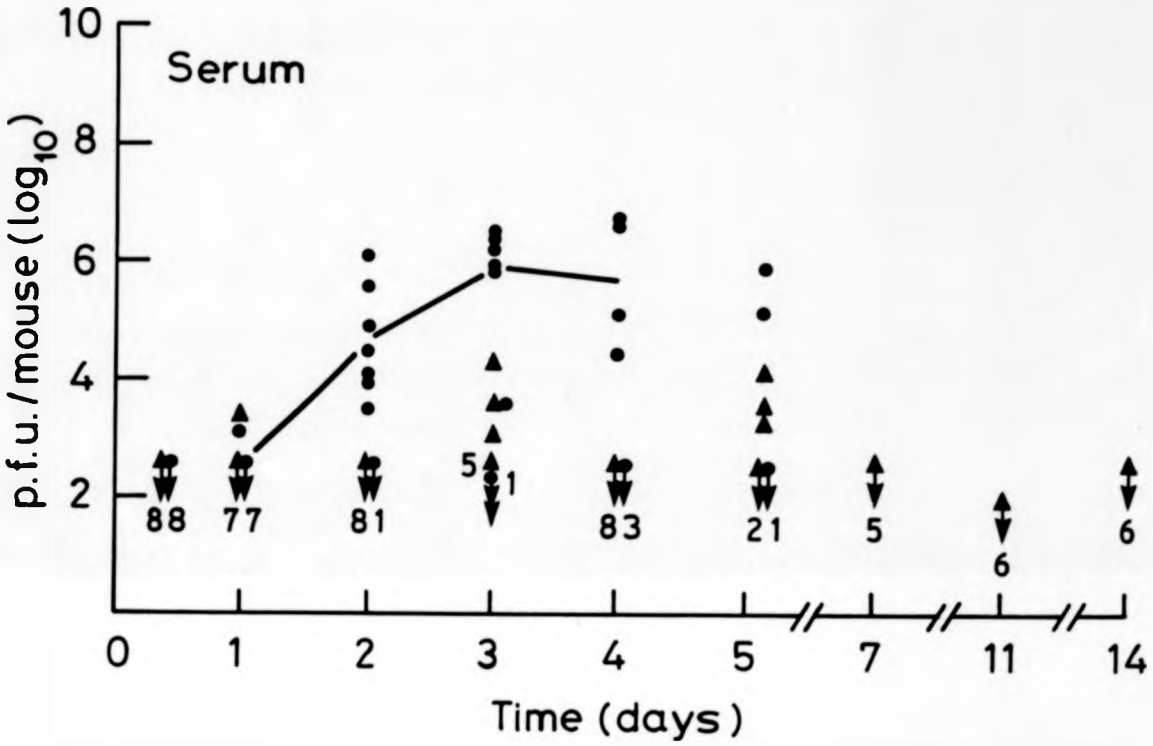

Olfactory lobes
p.f.u./mouse (log10)
10
8
6
4
2
0
8 8
5 2
3
3
7 2
3
1
6
6
0
1
2
3
4
5
7
11
14
Time (days)

Figure 19a Multiplication of SFV in mice inoculated with 10 LD_{50} and treated with UV DI SFV p13a - brain, and olfactory lobes

Mice were inoculated with 10 LD_{50} + UV SFV or 10 LD_{50} + UV DI SFV p13a and sacrificed at various times after infection. Curves represent mean infectivity titres of detectable virus for sample 10 LD_{50} + UV SFV. Arrows with adjacent number refers to the number of mice with undetectable infectivity. 10 LD_{50} + UV SFV ●; 10 LD_{50} + UV DI SFV p13a ▲.


Serum
p.f.u./mouse (log_{10})
10
8
6
4
2
88
77
81
5
1
83
21
5
6
6
0
1
2
3
4
5
7
11
14
Time (days)

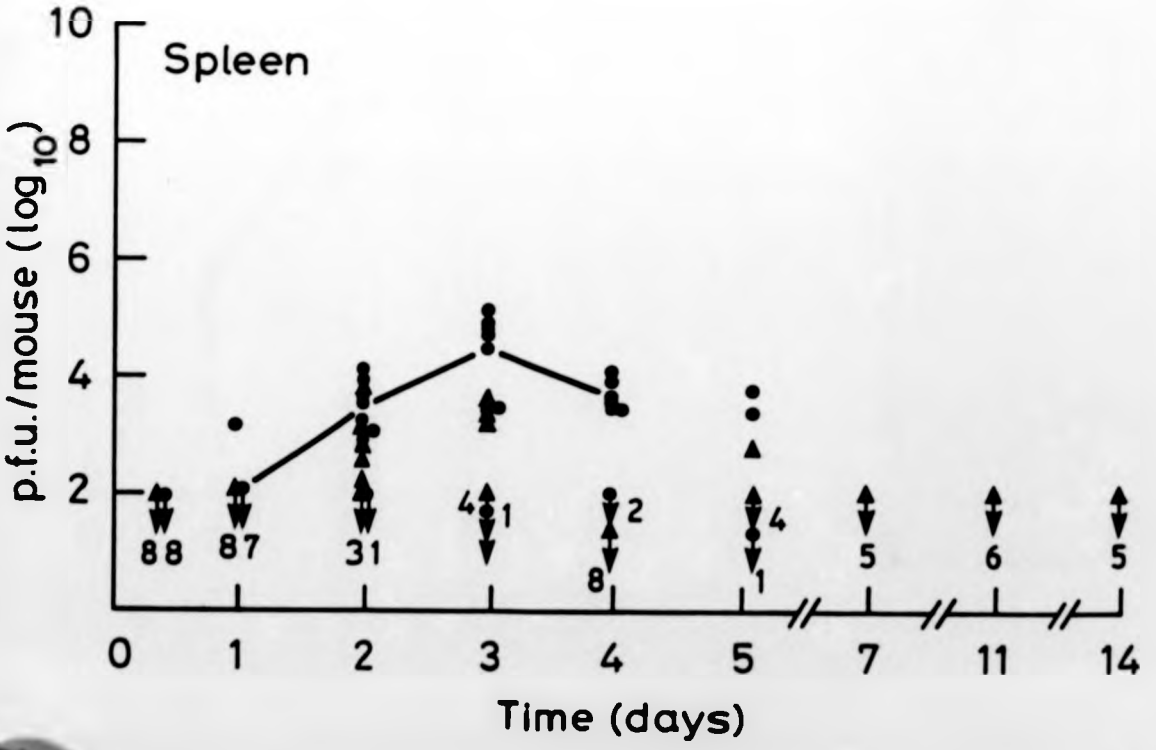

Spleen
p.f.u./mouse (log_{10})
10
8
6
4
2
88
87
31
4
1
8
2
1
4
5
6
5
0
1
2
3
4
5
7
11
14
Time (days)

Figure 19b. Multiplication of SFV in mice inoculated with 10 LD_{50} and treated with UV DI SFV p13a - serum, and spleen

Mice were inoculated with 10 LD_{50} + UV SFV or 10 LD_{50} + UV DI SFV p13a and sacrificed at various times after infection. Curves represent mean infectivity titres of detectable virus for sample 10 LD_{50} + UV SFV. Arrows with adjacent number refers to the number of mice with undetectable infectivity. 10 LD_{50} + UV SFV ●; 10 LD_{50} + UV DI SFV p13a ▲.

Table 31 Proportion of infectivity in tissues from mice treated with 10 LD_{50} + DI SFV p13a

Time p.i. (days)	Proportion of infectivity titres reduced in tissue sample†			
	olfactory lobes	brain	spleen	serum
0.5	0/8	0/8	0/8	0/8
1	5/8(-2)*	0/8	0/8	0/8
2	4/8	6/8	4/8(-1)	8/8(-1)
3	5/8	5/8	4/8(-1)	7/8(-1)
4	7/8	7/8(-2)	8/8	8/8
5	3/5	4/5	4/5(-1)	5/5(-1)

† No. mice with reduced infectivity/No. mice sampled.
Reduced infectivity is defined arbitrarily as ⩾99% reduction in infectivity compared to mean infectivity of mice treated with 10 LD_{50} + UV SFV. All samples after day 5 p.i. had reduced infectivities.

* number in bracket refers to number of mice from group infected with 10 LD_{50} + UV SFV which have "reduced"or no detectable infectivity.

Data from Fig. 19.

infectivity titres at all time points examined. There was little virus in serum (compared with other tissues) of DI virus-treated mice, but infectivity titres in the spleen on days 2 and 3 p.i. showed little reduction compared to mice which did not receive DI virus. These results demonstrate that DI virus not only prevents the encephalitis but also prevents spread of virus into other tissues. Inspection of infectivity titres of mice which comprise the group of individuals in which titres were reduced $\geqslant$99% showed that majority contained no detectable virus (brain 81%, olfactory lobes 92%, spleen 92%, serum 92% of mice sampled contained $\leqslant$400 pfu/tissue). At days 7, 11 and 14 p.i. no mouse had virus detectable ($\ll$400 pfu/mouse) in any of the tissues investigated. An exception was a single mouse which at day 14 had a brain infectivity titre of $10^{4.9}$ pfu, but none in the other three tissues examined.

e) Disproportionate reduction in infectivity titres in some tissues of some mice in which infections were modulated by DI SFV p13a

As discussed above, mice inoculated with DI SFV p13a fell into two classes (a) those with infectivity titres reduced by $\geqslant$99% compared with mice infected with 10 LD_{50} + UV SFV and (b) those with titres unaffected by inoculation of DI virus. In the majority of mice, the levels of infectivity in each of the four tissues investigated were either consistently low or consistently high, and the latter were indistinguishable from mice inoculated with 10 LD_{50} + UV SFV (Figure 17). However, 9 mice treated with DI virus p13a had infectivity titres which did not fit with either pattern. These had high titres in one or

more tissues and low titres in the others (Table 32). For example, mouse 6 on day 2 had no detectable virus in brain and serum, and yet there were high titres in the olfactory lobes and spleen, whereas no DI virus-treated mice (mouse 1 on day 2) had similar titres in the olfactory lobes and spleen, but $\geqslant 10^{5}$-fold more virus in the brain and $\geqslant 10^{1.8}$-fold more virus in the serum. It must be emphasised that only 9/62 (14.5%) had this unusual pattern of virus infection. This effect was only seen with DI virus p13a and none of the mice treated with DI virus p4 showed an unusual pattern of virus infectivity. Particularly noteworthy is the presence of virus in brain at 14 days after infection when one would have expected immune processes to have cleared virus completely.

f) Failure to detect DI virus in DI virus-treated mice

It was of obvious interest to follow the levels of DI virus in the above experiments. In an attempt to do so the YRA and RSIA were used as these appear to measure different parameters of interference. Both assays failed to detect DI virus in either brains or olfactory lobes of DI virus p4 or p13a treated mice. This result was unexpected since it has been demonstrated that inoculation of DI virus prevents death and inhibits virus multiplication. Possible explanations are that the assays may not be sufficiently sensitive (both the YRA and RSIA detect a minimum of $10^{5.15}$ DI particles/250 µl), that DI virus which interferes in mice does not register by these assays, or that DI virus is not propagated in the mouse. The latter suggestion would conflict with the results of Dimmock and Kennedy (1978) who detected DI virus in mouse

Table 32 Infectivity titres in tissues of individual mice inoculated with $10LD_{50}$ + UV DI SFV p13a*

Day p.i.	Mouse No.	Infectivity (log_{10} pfu/tissue)			
		brain	olfactory lobes	spleen	serum
2	1	7.3	6.9	3.2	4.4
	2	⩽2.0	⩽1.7	2.6	⩽2.6
	3	⩽1.7	5.7	3.0	⩽2.6
3	1	8.8	6.5	4.0	5.2
	2	⩽1.3	⩽2.0	3.6	4.3
	4	⩽1.3	5.2	3.0	⩽2.0
	6	4.0	4.2	3.3	3.1
	8	5.2	⩽1.7	⩽1.4	⩽2.6
5	1	7.5	5.7	2.8	4.6
	2	3.4	5.1	⩽2.0	4.1
	3	⩽1.6	⩽1.7	2.7	3.3
14	4	4.9	⩽1.7	⩽2.0	⩽2.0

* Data from Fig. 19.

brain after an amplification step. It would appear more likely that the apparent absence of DI virus is due to the insensitivity of the assays used.

To test these possibilities mice were co-inoculated with DI virus p8 plus 7 x 10^4 LD_{50}. Although DI virus p8 would not protect mice against this high dose of S virus the chance of co-infection, and therefore enrichment of DI virus, would be high and Figure 20 shows that DI virus p8 did not inhibit the multiplication of S virus. More importantly the interference assays (RSIA and YRA) failed to detect the presence of DI virus in any of the brain samples. No amplication assays were attempted. Thus the problem has not been resolved.

g) Protection of mice by mouse brain homogenate

To test the possibility that the DI virus propagated in brain does not register by in vitro assays, brain homogenates were used as a source of DI virus in mouse protection experiments. Preliminary experiments (Table 33) showed that co-inoculation of brain homogenate from uninfected mice, protected mice against 10 LD_{50} but not 100 LD_{50} S virus. Identical results were obtained by using 2 inoculations of brain homogenate 2 h apart. This non-specific effect was lost when brain was diluted 10^{-1} or more. Uninfected mouse brain homogenate protected cells against SFV challenge in in vitro interferon assays (see Methods) and protection was specific for mouse cells since hamster (BHK) cells were not protected (Table 34). This result was supported by the failure of uninfected mouse brain homogenate to inhibit SFV plaque formation in CEF

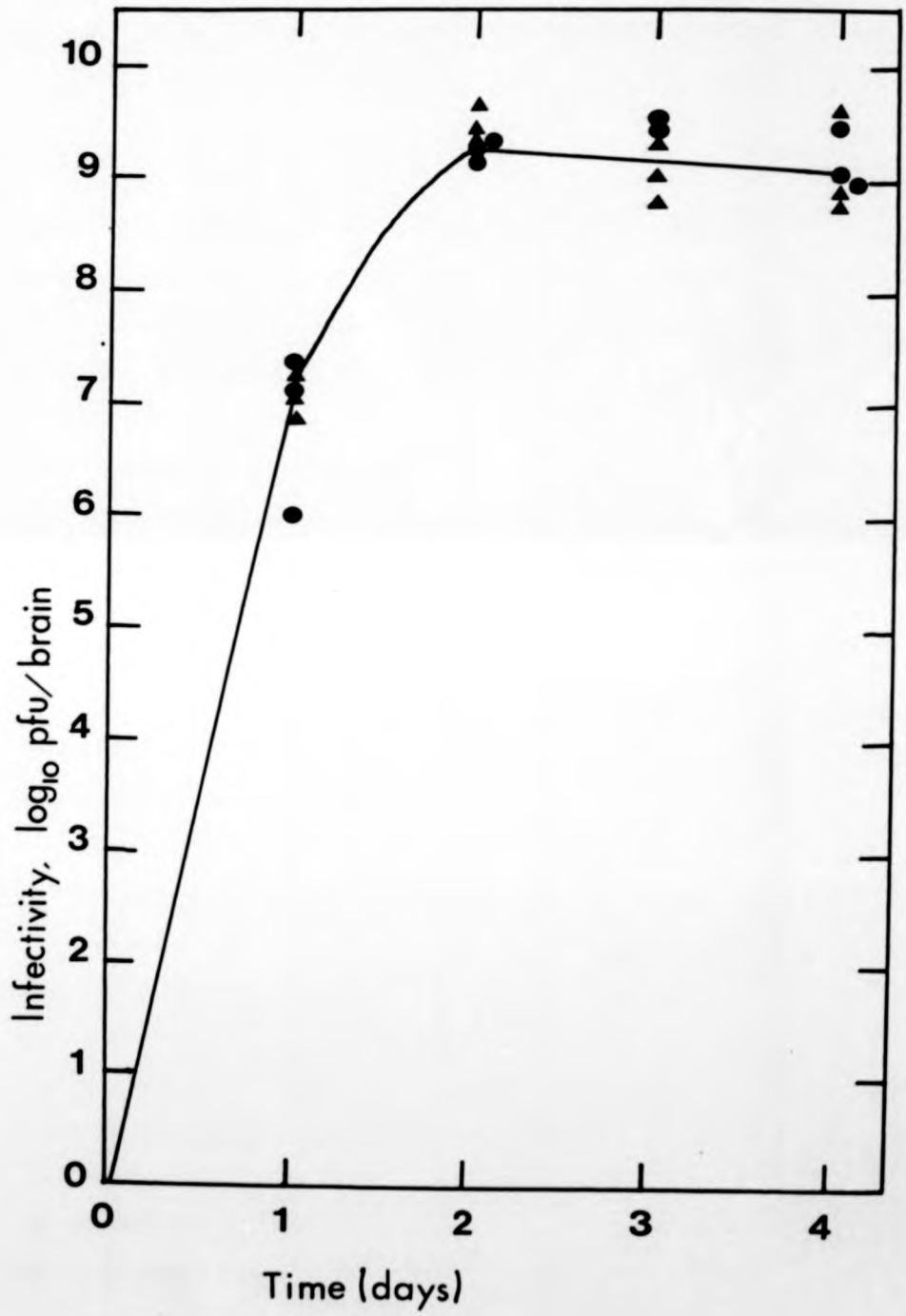

Infectivity, log10 pfu/brain
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
Time (days)

Figure 20. Growth curve of S SFV and S SFV plus pθ DI SFV in the brains of mice

Mice were inoculated by the i.n. route according to Methods. S virus was 7 x 10^4 LD_{50}. ▲ S virus + DI virus p8; ● S virus only.

Table 33 Protection of mice against SFV lethal encephalitis by administration of brain homogenate from uninfected mice

Inoculum†	Survivors	%
10 LD_{50}	1/10♠	10
10 LD_{50} + 1/1 MB	8/10	80
10 LD_{50} + 1/10 MB	1/10	10
10 LD_{50} + 1/100 MB	2/10	20
10 LD_{50}	0/10	0
10 LD_{50} + 1/1 MB	6/9	67
100 LD_{50}	0/10	0
100 LD_{50} + 1/1 MB	1/9	11

† Mice were inoculated by the i.n. route in a 20 µl volume. MB = uninfected mouse brain homogenate. Control mice inoculated with MB only survived without disease.

♠ No. surviving/no. inoculated.

Table 34 Assay for 'interferon' in uninfected mouse brain homogenate

Sample	Interferon titre† (Log_{10} units/ml)		
	L_{929} cells (- AMD)	L_{929} cells (+ AMD)	BHK cells (- AMD)
Uninfected mouse brain homogenate	2.7	3.2	≤1.0
mouse interferon (α/β)♣	3.1	≤1.0	≤1.0

† Interferon assay was performed either on mouse L_{929} or hamster BHK cells. Samples were inoculated either with or without 2 μg/ml actinomycin D (AMD). Interferon titres are expressed in arbitrary units.

♣ Mouse interferon was a standard prepared by induction of L_{929} cells with Newcastle Disease virus, and as such consists of a mixture of α and β interferon species.

cells. Thus the brain tissue debris was not directly "neutralising" infectivity. However, the protection factor was not interferon since actinomycin D did not inhibit protection. (Actiomycin D prevents depression of host cell genome by interferon by inhibiting host cell RNA synthesis). The nature of this protection factor is unknown but would appear to be similar to that reported by Woodward and Smith (1975) and Woodward et al. (1978).

As this non-specific protection diluted out at 1/10 dilution, the protecting abilities of brain homogenate from mice co-inoculated with 10 LD_{50} + DI SFV could nonetheless be tested. Infectious virus was removed from mouse brain by UV irradiation of 1/10 dilution of brain homogenate for 30 sec so that all infectivity was removed (UV inactivation kinetics of infectious virus in 1/10 dilution of brain homogenate were identical to that in tissue culture fluid: data not shown). Although uninfected mouse brain did not protect mice, small numbers (<30%) of mice were found to be protected against 10 LD_{50} by brain homogenate from mice originally treated with DI virus p4 or p13a (Table 35). This could therefore represent DI virus which did not register by in vitro assays as postulated above. However, brain homogenates from 10 LD_{50} + UV SFV infected mice also protected mice to the same extent. Mouse brains collected at 21 days p.i. from mice inoculated with 10 LD_{50} + DI SFV also protected mice as did brain homogenate from mice inoculated with DI SFV p13a only. On the other hand, brain homogenates from mice inoculated with avirulent SFV did not protect mice. This would indicate that brain homogenates will only protect against the homologous strain of SFV. Thus it is possible that the protection phenomenon is

Table 35 Protection of mice by brain homogenates from mice treated with 10 LD_{50} + DI virus, DI virus alone or avirulent SFV

Brain obtained from mice inoculated with:†	Day p.i. brain harvested	Virus titre in brain† (log_{10} pfu/brain)	Mice inoculated with 10 LD_{50} + 1/10 mouse brain from left hand section of table	
			Survivors†	Protection (%)
—			2/10	0
MBU‖			2/10	0
10 LD_{50} + p4	2.5	<3.6	3/10	10
10 LD_{50} + p4	"	<3.6	5/10	30
10 LD_{50} + p4	"	5.6	3/10	10
10 LD_{50} + UV SFV	"	6.9	6/10	40
10 LD_{50} + p13a	4	<3.6	1/10	0
10 LD_{50} + p13a	"	<3.6	1/10	0
10 LD_{50} + p13a	"	4.45	3/10	10
10 LD_{50} + p13a	"	3.9	3/10	10
10 LD_{50} + p13a	"	10.55	4/10	20
10 LD_{50} + UV SFV	"	10.6	5/10	30
—			0/10	0
10 LD_{50} + p4	21	<1.6	2/10	20
10 LD_{50} + p4	"	"	1/10	10
10 LD_{50} + p13a	"	"	2/10	20
10 LD_{50} + p13a	"	"	3/10	30
AV‖	"	"	1/10	10
AV	"	"	1/10	0
AV	"	"	0/10	0
p13a	"	"	4/10	40
p13a	"	"	4/10	40

† Groups of 10 mice were subsequently inoculated with this material UV irradiated for 30 sec to which 10 LD_{50} was added.
† Survivors = no. survivors/no. inoculated.
† Infectivity present in mouse brain before UV irradiation.
‖ MBU is uninfected mouse brain.
‖ Mice were inoculated with 8×10^4 pfu avirulent SFV.

due to DI virus but the presence of protection in brains from mice treated with 10 LD_{50} + UV SFV or DI virus only, and in brains collected at 21 days p.i. suggests that DI virus is stable in brain cells or some other, unknown, phenomenon is responsible for protection (which is not interferon or neutralising antibody).

Discussion

The above studies have shown that the DI SFV preparations, p4 and p13a, differ in the ways in which they inhibit the multiplication of S virus in the mouse. Control mice inoculated with 10 LD_{50} + UV SFV showed that infection was not modulated by the immunogenicity of the added non-infectious virus. Mice treated with DI SFV p13a had low infectivity levels throughout infection (except for spleen at 2 days p.i.), while DI SFV p4 treated mice have infectivity titres identical to non-treated mice for the first 2 days of infection. From day 3 post-infection only low infectivity levels were obtained. These results are a paradox since in the previous results section reported that mice treated with DI SFV p13a had strong immunity to challenge, while those treated with DI SFV p4 had weak immunity. Since mice treated with DI SFV p4 had the same distribution and amount of infectivity at day 2 p.i. as non-treated mice one would have expected there to have been sufficient immunogenic stimulus to mount a strong immune response to infection. Conversely, DI virus p13a-treated mice had reduced levels of infectivity throughout infection (apart from spleen) one might have expected immunity to be

impaired. Neither prediction was upheld by experimental observation. Two possible explanations are that DI SFV p13a treated mice synthesize sufficient non-infectious SFV antigen to be immunizing or that DI SFV p4 is immunosuppressive.

In an attempt to resolve the problem outlined above, mice were infected i.n. with different quantities of virulent or avirulent SFV to determine if it is possible to get undetectable levels of SFV in a mouse and yet to develop immunity to challenge. The results obtained are shown in Table 36. Inoculation of virulent SFV indicates that the LD_{50} is also the infectious $dose_{50}$ (ID_{50}) and that mice inoculated with less than 1 LD_{50} had no detectable virus in brain at day 4 p.i. and were susceptible to subsequent challenge with 100 LD_{50} at 21 days p.i. Inoculation of avirulent SFV resulted in similar virus titres in brain on day 4 p.i. (the peak day of infectivity) and all but one survived challenge with 100 LD_{50}. It is concluded that in a SFV infection where DI virus has not been inoculated, mice which have no detectable virus in brain develop no protective immunity and will succumb to challenge. Nonetheless, mice treated with 10 LD_{50}+ DI virus p13a must express sufficient SFV antigen to stimulate protective immunity but the nature and distribution of this SFV antigen is unknown at present.

Although both interference assays failed to detect DI virus in the brains of DI virus-treated mice, low levels of DI virus ($\leq 10^{5.15}$ DI particles) may be present in the mouse. Alternatively, the DI virus which interferes *in vivo* may not register in the *in vitro* assays. Indeed such interfering activity was induced in mouse brain after

Table 36 Characterisation of the response to challenge by 100 LD_{50} of mice previously inoculated with dilutions of virulent or avirulent SFV†

	pfu inoculated (log_{10})	pfu detected* (log_{10} pfu/mouse)	Survivors of primary infection‡	%	Survivors of challenge with 100 LD_{50} ‡	%
Virulent virus	2.8	≤1.6, 8.4, 8.3, 8.9	2/5	40	1/2	50
	1.8	all ≤1.6	5/5	100	1/5	20
	0.8	all ≤1.6	5/5	100	0/5	0
	1.8	all ≤1.6	5/5	100	0/5	0
Avirulent virus	6.6	7.1, 6.9, 6.5, 5.9	4/5	80	4/4	100
	5.6	6.5, 6.2, 6.2, 6.2	3/5	60	3/3	100
	4.6	6.4, 8.2, 6.2, 6.4	3/5	60	3/3	100
	3.6	6.4, 6.2, ≤1.3, 6.6	5/5	100	4/5	80
	2.6	3.0, ≤1.3, ≤1.3, ≤1.3	5/5	100	1/5	20
	1.6	all ≤1.3,	5/5	100	0/5	0
None‡			5/5	100	0/5	0

† Virus was inoculated using the i.n. route in a volume of 20µl according to Methods.

‡ No. surviving/No. inoculated.

‡ Mock infected with medium only.

* in the brains of mice at 4 days p.i.

infection. Further support for this category of DI virus arises from the data in Table 16 which shows that *in vivo* protection does not correlate with interference titres measured *in vitro*. These ideas would require there to be several different DI SFV genomes and indeed Kääriäinen *et al*. (1981) have shown DI SFV RNA to be heterogeneous.

Analysis of the infectivity titres in mice treated with DI SFV p13a revealed an anomolous distribution of infectivity in 9 individuals. Sampling means that we cannot know if these mice would have survived, possibly carrying a persistent infection which may have produced disease in later life (see next section). However, these results do indicate that besides resulting in the rapid clearance of virus from infected tissues, DI SFV can cause a more subtle modulation of infection.

Chapter 4

Persistence of Virus in Mice Protected by DI virus

Introduction

To date much has been reported of DI viruses being involved in the establishment and maintenance of persistent infections in tissue culture (for a review see Holland et al., 1980). Only a few studies, with arenaviruses and VSV, have been performed in vivo. In nature, arenaviruses cause persistent infections and it has been suggested that either these infections are autoregulatory, such that multiplication takes place without destroying infected cells, or that DI particles regulate infection (for reviews see Buchmeier et al., 1980 and Rawls et al., 1981). Fultz et al. (1982b) have shown that VSV persists in the LSH strain of hamster following co-inoculation of DI and S virus. Infectivity was detected up to 8 months after infection but only after co-cultivating for 1 to 4 passages.

In the previous chapter, infectious virus was found in the brain of one mouse at 14 days p.i. which had been protected by co-inoculation of DI virus p13a. As this appeared to be a persistent infection it was of interest to see if this result was representative of a general phenomenon. Thus a number of protected mice were examined for the presence of infectious virus at 12-21 days p.i. These times were chosen as mice not treated with DI viruses became ill on day 3 and died on day 5.

Results

a) Presence of infectious virus in the brains of protected mice

A total of 70 mice which had been protected by administration of DI virus p4 or p13a were sacrificed between days 12 and 21 p.i. Brains were removed and plaque assayed directly for infectivity (Table 37). Although all the mice were clinically healthy, 5 mice (16.1%) treated with 10 LD_{50} + DI virus p4, and 2 mice (5.1%) treated with 10 LD_{50} + DI virus p13a had infectious virus detectable in their brains.

It is difficult to find suitable controls for the above experiment since mice inoculated with 10 LD_{50} + UV SFV all die at day 5. However, mice were inoculated with an avirulent strain of SFV (A774) which at the dose used causes transient clinical signs of malaise. Mice recovered completely by day 10 p.i. (unpublished observations). Infectivity peaked in brain at day 4 to 6 p.i. and no mice sampled between 12 and 21 days p.i. had detectable infectious virus (0/32 or $<3\%$). The latest positive isolation of virus in brain (or elsewhere) was at 10 days p.i. This suggests that the isolation of infectivity from DI virus-treated mice, as shown in Table 37 is indicative of a persistent infection.

Brains containing detectable virus had infectivity titres which varied from totals of 4×10^1 to 4×10^5 pfu. No DI virus was detected in these mouse brains by either the RSIA or YRA *in vitro* interference assays. Since the 2 assays detect $\geq 10^{5.7}$ DIU/brain it is possible that low levels of DI virus are present. With low levels of infectivity in brain low levels of DI virus would be expected.

b) Properties of one isolate of infectious virus from the brain of a DI virus protected mouse

Virus isolated on day 14 from a mouse infected with 10 LD_{50} + DI virus p13a ($10^{4.9}$ pfu/brain; see Table 37) was examined in detail. This virus is coded I13a/14. Virus was passaged in BHK cells at a moi of 0.05. After incubating for 18 h at 37°C an infectivity titre of 1 x 10^4 pfu/ml was obtained. A repeat passage with 40 h incubation gave a titre of 2 x 10^6 pfu/ml. Since a number of workers have shown that infectious virus isolated from persistant infections has altered properties, such as tempeature senstivity and virulence (see review by Holland et al., 1980), I13a/14 was examined for any changes from the properties of the parental ts^+ S virus used in the inoculum.

Plaque formation by I13a/14 was neutralised by rabbit antisera against SFV showing that the virus was indeed SFV. The virulence of I13a/14 was compared to the original S virus inoculum (Table 38). Intranasal inoculation of 10 LD_{50} pfu-equivalents (6 x 10^3 pfu) of the I13a/14 isolate killed all the mice tested as did inoculation of 10 LD_{50} S SFV. All mice inoculated with 10 LD_{50} pfu-equivalents of avirulent SFV survived infection. Thus the isolate is at least as virulent as the parental virus.

Temperature sensitivity of plaque formation by I13a/14 was examined next to see if the virus had changed by multiplication in mice (Table 39). I13a/14 virus had the same plaque efficiency (39°C:33°C) as S virus. (S virus was known to be temperature sensitive [Barrett, 1980]). Plaque size and morphology were the same for both viruses. Thus, it would

Table 37 Presence of infectious virus in brains of mice protected by DI SFV or mice inoculated with avirulent SFV

Inoculum‡		Infectivity titre† (log_{10} pfu/brain)	Virus isolation✦	
day 12)		5.6, 2.5, 1.85, 1.6	4/12)	
day 13)	10 LD_{50} +		0/6)	5/31
day 14)	DI virus p4		0/5)	(16.1%)
day 21)		4.75	1/8)	
day 12)		1.6	1/14)	
day 13)	10 LD_{50} +		0/6)	2/39
day 14)	DI virus p13a	4.9	1/6)	(5.1%)
day 21)			0/13)	
day 12)			0/6)	
day 14)			0/6)	
day 15)	avirulent SFV		0/5)	0/32
day 16)			0/6)	(≤3.3%)
day 21)			0/9)	

† all infectivities were undetectable (≤1/6 pfu/brain) unless stated.
✦ no. mice with detectable virus/no. mice sampled.
‡ mice were inoculated by the i.n. route with DI virus as described in Methods or avirulent SFV (8×10^4 pfu).

Table 38 Lethality of virus isolated at 14 days p.i. (I 13a/14)

Inoculum†	Survivors✦
S virus	0/10
I 13a/14	0/10
avirulent SFV	10/10

† mice were inoculated i.n. with 6×10^3 pfu (= 10 LD_{50} S virus) in 20 µl. S virus is standard virus of the ts^+ strain of SFV.

✦ no. of mice surviving/no. of mice inoculated at 9 days p.i.

Table 39 Infectivity titrations of I 13a/14 virus and standard SFV at different temperatures

	Temperature (°C)	Titre (pfu/ml)	Efficiency of plaquing* %
Standard SFV	33	1.2×10^9	100
	39	2.0×10^7	1.7
I 13a/14 virus	33	1.5×10^6	100
	39	2.5×10^4	1.7

* titre 39°/titre 33°.

appear that the virus isolated from the brain is similar, if not identical to that used to inoculate the mice initially.

c) Neutralizing antibody levels in protected mice

Brain infectivities and neutralizing antibody titres present in the brain and sera of mice were taken on days 13 to 14 p.i. and were compared (Table 40). Although few (4/23) protected mice have high levels of neutralizing antibody in the serum ($\geq$1000) the mouse with infectivity in the brain was one of them. It had no detectable antibody in brain. Therefore serum neutralizing antibody titres did not correlate with the presence or absence of infectivity in the brain. Mice infected with avirulent SFV had high serum neutralizing antibody titres but no detectable infectivity in the brains. Few protected mice had neutralizing antibody titres in brain and these did not correlate with high serum neutralizing antibody titres. Generally, avirulent SFV infected mice had significant neutralizing antibody in brain.

Discussion

Infectious virus was isolated from the brains of a minority (10%) of DI SFV-protected mice examined between 12 and 21 days p.i. Infectivity was isolated from more mice treated with DI virus p4 (16%) than those treated with DI virus p13a (5%). The greater number of isolations from DI virus p4 treated mice may correlate with their weak adaptive immune

Table 40 Brain infectivities and neutralizing antibody titres in mice at 13-14 days p.i.

Inoculum‡		Brain infectivity‡ (pfu/mouse)	Neutralizing antibody titre† brain	serum
Avirulent	1	all $\leqslant$1.6	$\leqslant$9	3162
	2		16	1778
	3		35	891
	4		89	1584
	5		158	1000
10 LD_{50} + DI virus p4	1	all $\leqslant$1.6	all $\leqslant$9	$\leqslant$2
	2			$\leqslant$5
	3			$\leqslant$9
	4			$\leqslant$9
	5			$\leqslant$20
	6			$\leqslant$2
	7			9
	8			32
	9			2000
	10			2000
	11		N.D.	3162

Table 40 (continued)

Inoculum♣		Brain infectivity♣ (pfu/mouse)	Neutralizing antibody titre† brain	serum
10 LD_{50}	1	all ⩽1.6	⩽9	⩽9
+ DI	2		⩽9	⩽9
virus	3		⩽9	⩽9
p13a	4		⩽9	9
	5		⩽9	10
	6		⩽9	18
	7		⩽9	22
	8		⩽9	398
	9	4.9	⩽9	1000
	10	⩽1.6	14	141
	11	⩽1.6	14	501
	12	⩽1.6	N.D.	22

† Results taken from Table 20.

♣ Results taken from Table 37.

♣ Mice were inoculated with avirulent SFV (8×10^4 pfu) or 10 LD_{50} + DI virus as described in Methods.

N.D. = not done.

response. Although isolations were made at a comparatively short time (12-21 days) after infection there is no doubt that the virus is persisting relative to non-treated mice which die on day 5 or avirulent SFV-infected mice which do not yield infectious virus after day 10. Whether or not this is a "persistent infection" depends on how this is defined. Protected mice were clinically healthy throughout all experiments and a group of 12 mice protected by DI SFV p13d have been observed up to 85 days p.i. and have remained healthy throughout this period. Thus there is no evidence of a persistent infection leading to disease. However, if only 10% mice are persistently infected such a small group of mice would not be expected to show clinical signs of disease.

Since infectious virus could be directly isolated from protected mice, co-cultivation techniques such as required by Fultz et al. (1982b) to detect VSV under similar experimental conditions were not used, so it is possible that infectious virus may be present in a higher proportion of protected mice. Only one isolate was compared with the inoculum virus and did not differ in temperature sensitivity characteristics and lethality. This contrasts with the results of Fultz et al. (1982b) who found that the viruses isolated from hamsters were temperature sensitive, small plaque mutants. It is intriguing that a mouse can remain clinically healthy with a high level of virulent virus in its brain. Perhaps the infectious virus is localised to a specific area, possibly in equilibrium with an undetectable amount of DI virus. This mouse had a high level of serum neutralizing antibody, but no detectable neutralizing antibody in the brain. Clearly serum neutralizing antibody is ineffective at clearing infectivity.

Chapter 5

Modulation of SFV infection by DI SFV after intraperitoneal inoculation

Introduction

In the General Introduction it was shown that the majority of studies of DI viruses in animals have involved infections of the central nervous system after either i.c. or i.n. inoculation. There have been only three reports on the prophylactic effects of DI particles inoculated by other routes. Early studies by Mims (1956) on Rift Valley fever virus in mice showed that intravenous inoculation of "incomplete" virus protected mice against i.c. inoculation of S virus. Recently, Fultz et al. (1982a) reported that hamsters could be protected against a lethal VSV infection, initiated by i.p. inoculation, by biologically active DI particles, also inoculated by the i.p. route. However, there was also heterologous interference by DI particles of the New Jersey serotype of VSV and induction of interferon by DI VSV in mice, suggesting that protection was not due to the intrinsic interfering ability of DI virus. Smith (1981) has proposed that "interfering virus" is involved in the genetic resistance of mice to the flavivirus, Banzi, since after i.p. inoculation, mice which were resistant to infection had high levels of "intefering virus", while susceptible strains of mice had much lower titres.

This section reports on the ability of DI SFV to prevent the lethal encephalitis in mice initiated by SFV inoculated by the i.p. route. DI SFV was also inoculated i.p.

Results

a) Defective interfering Semliki Forest virus preparations

Details of the passage histories, haemagglutination and interference assay titres of the DI SFV preparations used in the experiments are shown in Table 41. In addition to DI virus these preparations also contained between 10^6 and 10^8 pfu/ml infectious virus. S virus was inactivated by UV irradiation as before, and the length of exposure was chosen to ensure that the irradiated DI virus preparations contained no infectivity detectable by plaque assay (Table 41). Thus DI virus preparations used in this section of the work were exactly the same as those in the experiments using the i.n. route of inoculation.

b) Determination of a sub-immunogenic dose of SFV antigen

In order to analyse the modulation of SFV infection in mice by DI SFV it was necessary to distinguish between the intrinsic interfering properties of the DI virus and its effect as an immunogen. The total amount of SFV antigen in DI virus preparations was determined by haemagglutination and the DI SFV preparations used contained a maximum antigenic mass of 4 HAU/ml. Non-infectious SFV antigen, prepared as previously, by UV irradiating S SFV, was diluted to a concentration of 4 HAU/ml, and used as a control for the immunogenic effects of DI virus. Such UV SFV antigen did not alter the course of disease caused by 10 LD_{50} S virus and did not reduce its lethality (Table 42). This control also excluded the possibility that the DI virus was preventing infection by blocking attachment to cellular receptor sites.

Table 41 Properties of DI SFV preparations

DI SFV preparation				Interference titre (DIU/ml)			
Passage history	Antigenic mass (HAU/ml)	Infectivity (pfu/ml)	Time of UV (sec)†	RSIA		YRA	
				Before UV	After UV	Before UV	After UV
p4 (B4)*	4	1×10^8	100	56	8	126	⩽4
p6 (B6)	⩽4	1×10^7	100	316	⩽4	501	⩽4
p13a (B13)	4	3×10^7	60	50	6	112	8
p23 (B21 C2)	⩽4	2×10^6	60	20	⩽4	89	⩽4

* The designation indicates that this DI virus preparation has had four undiluted passages (p) all in BHK (B) cells. Lower case letters indicate sister stocks. Passage in chick embryo cells is indicated by C.

† This treatment left no infectivity detectable by plaque assay.

Table 42 Effect of SFV antigen on lethality in mice of 10 LD_{50} S virus after i.p. inoculation

Inoculum†	Survivors†	Mean day of death‡
10 LD_{50}	1/10	6.61
10 LD_{50} + UV SFV	1/10	6.85

† Mice were inoculated with 10 LD_{50} diluted in medium or 10 LD_{50} diluted in UV SFV. UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml.

♣ Number of surviving mice/number of mice inoculated on day 14 p.i.

‡ Mean day of death is the sum of the day on which each mouse died divided by the number of mice.

c) Modulation of SFV-induced disease by administration of DI SFV

The encephalitis in mice after i.p. inoculation and the disease induced in adult CFLP mice inoculated with 10 LD_{50} followed a reproducible pattern. Mice remained apparently healthy until 5 days p.i. and then showed signs of malaise (ruffled fur and inactivity), which was followed within 24 h by paralysis of the hind limbs. This progressed to complete paralysis and by day 8 p.i. most mice were dead. Usually with 10 LD_{50}, 90% of mice die and the remainder appear to have a silent infection which is nevertheless immunizing (see Table 47).

Mice were inoculated with a mixture of 10 LD_{50} S virus together with DI virus p13a; controls received 10 LD_{50} plus non-infectious UV irradiated S virus (UV SFV; 4 HAU/ml) as described above. Table 43 shows that DI SFV p13a modulated the course of the disease by retarding the onset of clinical signs, and the progression through paralysis to death. For example, at 6.75 days p.i. 67% mice infected with 10 LD_{50} + UV SFV were dead compared with 11% of those inoculated with 10 LD_{50} + DI SFV p13a. The mean time of death for 10 LD_{50} + UV SFV ws 6.6 days and this was extended to 9.2 days for 10 LD_{50} + DI SFV p13a. Overall inoculation of mice with 10 LD_{50} + DI SFV p13a resulted in more than double the number surviving inoculation with 10 LD_{50} + UV SFV. Control mice inoculated with only UV SFV or only DI SFV p13a remained healthy.

d) Comparison of the ability of different DI SFV preparations to modulate SFV-induced disease

It is not possible to purify DI SFV from S SFV, therefore quantitative

Table 43 Clinical observations of DI SFV treated mice

Inoculum	Clinical symptom	Day post infection 3.75	4.75	5.75	6.75	7.75	8.8	9.8	11.1	20.8	survivors (%)	Protection‡ (%)	Mean day of death
10 LD_{50} + UV SFV*	Dead† complete paralysis hind limb			2	6	7							
	paralysis malaise			1	1								
	well		1	3									
		9	8	3	2	2	2	2	2	2	22	0	6.6
10 LD_{50} + DI SFV†	Dead† complete paralysis hind limb				1	2			4				
	paralysis						1	1					
	malaise			1	1	1							
	well			1	1	1	1	1					
		9	9	7	6	5	5	5	5	5	55	33	9.2

* UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml.
† UV DI SFV p13a.
† cumulative total
‡ The percentage of mice which survived through inoculation of DI virus was corrected by subtraction of the percentage surviving 10 LD_{50} + UV SFV only.

comparisons between DI SFV preparations are difficult. Since DI SFV preparations contain different quantities of antigen, infectivity and interference titres by *in vitro* assay (see Table 41), only qualitative comparisons between DI SFV preparations can be described.

The ability of DI SFV preparations to protect mice after i.p. inoculation is shown in Table 44. All 4 DI SFV preparations tested modulated the disease to some extent but this appeared to have two manifestations: mice survived the infection and showed no signs of disease, and up to 51.5% were protected in this way (DI SFV p6). Secondly, death was delayed over the mean day of death for 10 LD_{50} + UV SFV of 6.7 days. All DI SFV preparations (except p4) delayed death significantly. There appears to be no correlation between the extent of delay in death and number of survivors for DI SFV-treated mice. For instance, both DI SFV preparations p4 and p13a modulated the disease to some extent and similar numbers of mice survived (27 and 22% respectively) without signs of disease. However, these two DI viruses differed in their ability to modulate infection by delaying death. DI SFV p13a delayed death by 1.5 days, while mice treated with DI SFV p4 died at the same time as controls inoculated with 10 LD_{50} + UV SFV. Similarly, although DI virus p6 protected 51.5% it only delayed death in the others by 1.7 days, a figure close to that seen with DI virus p13a (1.5 days) which protected much less efficiently (22%). Thus, (a) survival from infection without showing signs of disease and (b) delay in death may reflect different mechanisms of interference. There was no correlation between either *in vitro* interference assay (RSIA or YRA) and either of the above effects of DI virus inoculated i.p.

Table 44 Protection of mice against a lethal SFV encephalitis by administration of different DI SFV preparations

Inoculum	% survivors‡ (± S.E.M.)	Mean day of death (± S.E.M.)	Delay in death* (days)
10 LD_{50} + UV SFV†	0 (n = 7)	6.7 ± 0.12	0
10 LD_{50} + DI virus p4†	27 ± 1.91 (n = 4)	6.8 ± 0.29	+ 0.1
10 LD_{50} + DI virus p6	51.5 ± 0.50 (n = 2)	8.4 ± 0.15	+ 1.7
10 LD_{50} + DI virus p13a	22 ± 2.00 (n = 4)	8.2 ± 0.34	+ 1.5
10 LD_{50} + DI virus p23	20 ± 0.00 (n = 2)	8.0 ± 0.12	+ 1.3

† UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml. Details of DI virus preparations are in Table 41.

‡ The percentage of mice which survived through inoculation of DI virus was corrected by substraction of the percentage surviving 10 LD_{50} + UV SFV only. n refers to the number of experiments performed. Each experimental group contained 9 or 10 mice.

* Compared to the mean day of death of mice infected with 10 LD_{50} + UV SFV.

e) Effect of inoculation of DI virus prior to and after infection with S SFV

DI SFV was administered to mice i.p. at different times relative to i.p. inoculation of 10 LD_{50}, to determine if this affected modulation of the lethal encephalitis. Post-inoculation did not protect, while pre-inoculation protected no better than co-inoculation with DI virus when compared to control mice inoculated with UV SFV.

f) Effect of Myocrisin on the ability of DI SFV to modulate S SFV infection

Macrophages are thought to play an important role in host immune mechanisms, particularly in the peritoneal cavity (Mogensen, 1979) and this role has also been demonstrated for SFV infections after i.p. inoculation (Allner et al., 1974; Bradish et al., 1975; Oaten et al., 1980; Mehta and Webb, 1982; Pathak and Webb, 1983). Macrophage activity can be diminished by treatment with colloidal gold salts such as Myocrisin (sodium auro-thio-malate) and this procedure has been used to determine if macrophages were contributing to the DI SFV-mediated modulation of SFV encephalitis. Table 45 confirms previous studies (Allner et al., 1974; Oaten et al., 1980) that Myocrisin enhances the lethality of the 10 LD_{50} dose by reducing the mean day of death. Death of mice treated with DI SFV in the presence of Myocrisin was delayed by 1.5 days, from 5.4 to 6.9 days p.i. However, there were no survivors, suggesting that under these conditions macrophages are required for survival.

The LD_{50} of SFV in mice treated with Myocrisin was 3×10^2 pfu compared

Table 45 **Effect of Myocrisin upon the ability of DI SFV to modulate SFV infection in mice**

Inoculum	No. surviving / No. inoculated	% survivors	Mean day of death
10 LD_{50} + UV SFV†	1/10	20	7.0
10 LD_{50} + DI virus p13a✦	3/10	30	7.9
10 LD_{50} + UV SFV + Myocrisin‡	0/10	0	5.4
10 LD_{50} + DI virus p13a + Myocrisin	0/10	0	6.9

† UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml.

✦ DI SFV p13a was UV irradiated for 60 sec before use.

‡ Myocrisin was administered as described in Methods.

Table 46 Ability of DI SFV to modulate SFV infection using the low pfu:LD_{50} ratio found in Myocrisin-treated mice

Inoculum	No. surviving / No. inoculated	% survivors	Mean day of death
10 LD_{50} + UV SFV†	2/10	20	6.9
10 LD_{50} + DI virus p13a‡	4/10	40	8.3
10 MLD_{50}♣ + UV SFV + Myocrisin	3/10	30	7.2
10 MLD_{50}♣ + DI virus p13a + Myocrisin	7/10	70	N.A.*

† UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml.

♣ 10 (Myocrisin)LD_{50} is 3×10^3 pfu. In control mice there are 1.25×10^5 pfu/LD_{50}.

‡ DI SFV p13a was UV irradiated for 60 sec before use.

* N.A. = not applicable, only 3 mice died.

with 1.25 x 10^5 pfu in control mice. Using 10 (Myocrisin) LD_{50} SFV there was the same proportion of survivors as in non-Myocrisin treated mice, but no difference in the mean day of death (Table 46). This experiment suggests that modulation of infection by DI virus is mediated partly through DI virus itself and partly via macrophages.

g) Effect of administering DI virus and S SFV by different routes

Since the encephalitis caused by i.n. inoculation of SFV can be prevented by i.n. inoculation of DI SFV it was of interest to see if i.n. administration of DI virus would protect mice against the encephalitis resulting from i.p. inoculation of S virus. DI SFV p13a was administered intranasally in two inoculations each of 20 µl, one two hours before and the other at the same time as the 10 LD_{50} were inoculated into the peritoneal cavity. DI virus inoculated by the i.n. route failed to modulate the disease caused by S virus after i.p. inoculation and no delay in death was observed. It should be remembered that only 40 µl DI virus was administered by the i.n. route, while 100 µl was inoculated i.p., so that direct comparisons between the two routes cannot be made. Administration of DI virus by the i.n. route at later times relative to i.p. inoculation of S virus were not examined.

h) Challenge of DI SFV-protected mice by S virus at 21 days after the initial infection

Mice which had survived the initial infection were challenged with 100 LD_{50} S virus at 21 days p.i. to determine if the mice had developed

solid protective immunity in response to the initial infection. All other survivors were also challenged. Table 47 shows that all DI virus protected mice were resistant to challenge. However, the majority of mice inoculated with DI virus alone or non-infectious UV SFV alone generated a protective immune response (although of course this was insufficient to prevent the initial infection). DI SFV p23 only protected 44% mice challenged. In comparison, the majority of mice inoculated with DI virus or UV SFV only by the i.n. route were susceptible to challenge (Table 17). However, it should be remembered, a smaller amount of virus is inoculated by the i.n. route. The result for DI virus p4 + 10 LD_{50} is in contrast to that obtained after i.n. inoculation where mice are protected in the absence of an adaptive immune response (see Table 17). Presumably the difference may reside in the efficiency of DI virus p4 as an immunogen by the i.p. route.

i) Production of infectious virus in DI SFV-treated mice

Since DI virus can delay the mean day of death or prevent disease and death in a proportion of animals, it was of interest to see how levels of infectivity were affected. SFV is neurotropic and causes an encephalitis after i.p. inoculation and in CFLP mice SFV reaches peak infectivity titres in the brain at day 5 p.i. (unpublished observations). Mice were infected with 10 LD_{50} + UV SFV or 10 LD_{50} + DI SFV p13a, killed at day 5 p.i. and infectivity titres in the brain, spleen and serum determined. Table 48 shows that there was low or no detectable levels of infectivity in the sera and spleens in mice of both groups. Comparison of infectivity titres in brain showed that there was

Table 47 Ability of mice which had survived the infection with 10 LD_{50} SFV by treatment with DI SFV preparations to survive a second i.p. inoculation of 100 LD_{50} SFV†

First inoculum	Survivors	
	No. survivors / No. inoculated	%
10 LD_{50} + UV SFV	15/15	100
Uninfected	0/16	0
UV SFV	12/14	86
DI virus p4	6/6	100
10 LD_{50} + DI virus p4	14/14	100
DI virus p6	5/5	100
10 LD_{50} + DI virus p6	6/6	100
DI virus p13a	8/10	80
10 LD_{50} + DI virus p13a	12/12	100
DI virus p23	4/9	44
10 LD_{50} + DI virus p23	6/6	100

† at 21 days p.i.

Table 47 Ability of mice which had survived the infection with 10 LD_{50} SFV by treatment with DI SFV preparations to survive a second i.p. inoculation of 100 LD_{50} SFV†

First inoculum	Survivors	
	No. survivors / No. inoculated	%
10 LD_{50} + UV SFV	15/15	100
Uninfected	0/16	0
UV SFV	12/14	86
DI virus p4	6/6	100
10 LD_{50} + DI virus p4	14/14	100
DI virus p6	5/5	100
10 LD_{50} + DI virus p6	6/6	100
DI virus p13a	8/10	80
10 LD_{50} + DI virus p13a	12/12	100
DI virus p23	4/9	44
10 LD_{50} + DI virus p23	6/6	100

† at 21 days p.i.

no significant difference between the two groups. In 27% (3/11) of the DI SFV treated group and 22% (2/9) of the 10 LD_{50} + UV SFV group no virus was detected. Brain infectivity titres were the same with means of $10^{6.01} \pm 10^{1.57}$ (1 s.e.m.) pfu/mouse in DI SFV-treated mice and $10^{5.57} \pm 10^{1.59}$ pfu/mouse in non-treated mice. In the control experiment, 36% (4/11) of DI SFV treated mice survived compared to 16% (2/12) of non-treated mice, and there was a delay in death of 1.5 days. This presents a paradox since infectivity titres are similar, but there was both significant delay in death and double the number of survivors in DI SFV treated mice.

j) Production of DI virus in mice inoculated with DI SFV and S virus

Assays for DI SFV by the RSIA and YRA were performed on the mice described above. Using the RSIA, DI virus was present in 4/11 sera of DI SFV-treated mice, whereas all the sera of mice inoculated with 10 LD_{50} + UV SFV were negative (Table 48). Insufficient sera remained for YRAs. No DI virus was detected in brain or spleen samples from either group of mice. Why DI virus was detected in serum where there was little infectious virus (Table 48) and not in the brain is unknown.

Discussion

Evidence has been presented that DI SFV preparations inoculated i.p. are able to modulate the course of infection initiated by i.p. inoculation of S virus. Antigen controls showed that protection was not due to the

Table 48 Virus titres in mouse tissues at day 5 p.i. after i.p. administration of 10 LD_{50} + DI SFV p13a

Inoculum† (mouse number)		Infectivity (log_{10} pfu/mouse♣)			Interference titre‡ (log_{10} DIU/mouse♣)
		Brain	Spleen	Serum	Serum
10 LD_{50} + UV SFV	1	≤1.00	≤1.81	≤1.60	≤1.2
	2	5.90	≤0.70	≤2.60	≤1.2
	3	4.45	1.18	≤1.60	≤1.2
	4	6.87	≤0.70	≤1.60	≤1.2
	5	5.90	1.30	2.08	≤1.2
	6	≤1.00	≤0.70	1.90	≤1.2
	7	1.95	2.20	2.78	≤1.2
	8	7.60	≤0.70	≤1.60	≤1.2
	9	6.34	1.70	2.60	≤1.2
10 LD_{50} + DI virus p13a	1	7.60	≤0.70	≤2.60	≤1.2
	2	7.95	1.30	≤2.60	2.6
	3	7.90	1.30	≤2.60	≤1.2
	4	6.54	≤0.70	2.64	≤1.2
	5	≤1.00	1.74	≤1.60	≤1.2
	6	6.08	≤0.70	≤1.60	3.1
	7	4.41	≤0.70	2.08	2.8
	8	≤1.00	0.70	≤1.60	≤1.2
	9	≤1.00	≤0.70	≤1.60	≤1.2
	10	5.00	≤0.70	1.60	≤1.2
	11	2.60	≤0.70	≤1.60	3.4

† UV SFV is non-infectious UV irradiated S virus at a concentration of 4 HAU/ml. DI SFV p13a was UV irradiated for 60 sec before use.

♣ For calculations of virus titre/mouse the volume of the brain is assumed to be 0.3ml, spleen 0.2ml, and serum 4 ml (Kaliss and Pressman, 1950).

‡ Obtained by the RSIA. DI virus was only detected in sera.

immunogenicity of the DI SFV or its ability to block the attachment of infectious virus to cell surface receptors.

In these experiments up to 51.5% of mice survived a lethal infection through administration of DI SFV. The data presented shows that DI SFV is also capable of delaying both the onset of disease and the mean day of death of mice inoculated with 10 LD_{50} SFV. The extent to which DI SFV modulates infection as judged by both mean day of death and proportion of survivors varied between different DI SFV preparations. For example, similar numbers of mice could be protected with either no delay in death (e.g. DI virus p4) or delay in death (e.g. DI virus p13a). As with the i.n. results, no correlation between *in vitro* interference titre and protection of mice was observed.

The data do not explain how DI virus modulates the lethal encephalitis since infectivity titres in the brains of mice inoculated with 10 LD_{50} + DI SFV are as high as in control mice infected without DI virus. However, DI virus was detectable in the serum of DI virus-treated mice. It is possible that brains contain low quantities of DI virus which are undetectable by *in vitro* assay and if so, these may act by stimulating local host immune responses. The detection of DI virus in the sera recalls the findings of Smith (1981) who reported that "interfering" virus originates in cells of the lymphoreticular system. The results also indicate that host immune responses are involved in DI SFV-induced modulation of infection since macrophages are required for maximum protection (Table 45).

Comparison of DI virus modulation of SFV infection initiated by the i.p. route and the i.n. route reveals a number of differences. Firstly, administration of DI virus by the i.n. route did not alter the time of death and secondly, some DI virus preparations which modulated infection when inoculated by the i.p. route were inactive by the i.n. route (Table 49). For example, DI SFV p6 protected 51.5% mice inoculated intraperitoneally but none by the i.n. route. Also, DI virus p6 protected more mice than DI viruses p4 and p13a by the i.p. route, while the reverse applies by the i.n. route. This may indicate that, protection of mice by DI SFV inoculated by the i.p. route occurs by a mechanism different from that operating after i.n. inoculation. This idea is supported by the observations that i.n. inoculation of DI virus inhibits multiplication in brain (Figs. 18 and 19), whereas i.p. administration had no affect on brain infectivity (Table 48). This may mean that DI SFV preparations stimulate different host functions depending on the route of inoculation. However, it should be remembered that different quantities of DI virus have been inoculated by the 2 routes and this may be partly responsible for the results obtained.

Table 49 Summary of the protection of mice against a lethal encephalitis by administration of various DI SFV preparations

DI SFV preparation	i.p. inoculation† Survivors (%)	i.p. inoculation† Delay in death (days)*	i.n. inoculation‡ Survivors (%)
p4	27	+ 0.1	64
p6	51.5	+ 1.7	0
p13a	22	+ 1.5	59
p23	20	+ 1.3	0

† Details of experiments are taken from Table 44.

‡ Results are taken from Table 16.

* There was no delay in death with i.n. inoculation.

General Discussion

The majority of work on DI particles has involved tissue culture studies. These have led to the suggestion that DI viruses have a role in modulating both acute and persistent virus infections in nature (for a review see Holland et al., 1980). Unfortunately, few animal studies have been carried out to investigate these ideas.

In this thesis it has been shown that DI SFV preparations can prevent the lethal encephalitis in mice which follows i.n. inoculation of S SFV. Not only was death prevented, but mice remained clinically healthy (i.e. they were "protected" from disease). These experiments were carefully controlled and it was shown that the immunogenic load of virus was not the cause of protection. The above results confirmed those obtained by Dimmock and Kennedy (1978). Few studies on other viruses have shown the same extent of protection as we have obtained with the SFV system, the exceptions being Welsh et al. (1977) and Jones and Holland (1980) using lymphocytic choriomeningitis virus and VSV and their DI viruses respectively. The results from experiments with DI SFV have been obtained using DI viruses from unconcentrated tissue culture fluids and this contrasts with the studies using VSV and lymphocytic choriomeningitis virus, where large numbers of DI particles were required for protection.

The multiplication of SFV in mice treated intranasally with DI virus showed that, in general, infectivity was greatly reduced in most tissues throughout infection (see below for exceptions). Thus DI virus limits the spread of virus throughout the animal and infectious virus is rapidly eliminated. In a few mice an altered pattern of infectivity was seen in which infectivity titres were reduced in some, but not all of

the tissues examined. It would appear that DI virus can cause subtle tissue specific affects in a minority of DI virus-treated mice. The detection of infectious virus in the brains of a few protected mice between 12 and 21 days p.i. suggests that DI virus is modulating the acute infection and causing it to become persistent. Before these experiments SFV was considered only to cause acute virus infections in vertebrates and it had not been reported to cause persistent infections. Fultz et al. (1982b) have produced the only other study reporting a persistent infection in animals initiated by DI virus, using VSV in hamsters. However, VSV was recovered only after co-cultivation of brain, while SFV differs as virus can be directly isolated from brain. The low level (10%) of "persistence" makes experimental observations difficult and clearly more mice need to be sampled and later time points after infection examined. Co-cultivation of brains with no detectable infectivity is required before the results can be fully evaluated. Nonetheless, the SFV system may provide an excellent model for studying the role of DI viruses in persistent virus infections.

The prevention of acute SFV infections in mice by DI virus supports the suggestion that DI viruses play a role in modulating virus infections in nature. Possibly DI viruses are involved in recovery from virus infections since they would cause a reduction in levels of S virus in the infected hosts. Alternatively, the presence of DI virus may cause a virus infection to be sub-clinical rather than manifesting signs of disease. The data obtained sheds no light on the role of DI virus in maintaining persistent virus infections. Future studies are required in this direction. In view of the results obtained with DI SFV in mice, a role for DI viruses, may be speculated for persistent virus infections

in nature. For example, virus antigens or particles can be detected in the neurological diseases subacute sclerosing panencephalitis and multifocal leukoencephalopathy in man and visna in sheep, while other persistent infections cause minimal damage to the host (e.g. cytomegalovirus, polyoma-like (JC and BK) and papilloma viruses in man, and lactic dehydrogenase virus and lymphocytic choriomeningitis virus in mice). At the moment all this is speculation and will only be understood with further studies.

Evidence has also been presented that DI SFV preparations are biologically heterogeneous and the various parameters of heterogeneity are described in Table 50. The ratio of interference titres by two in vitro interference assays (RSIA and YRA) differed between preparations, as did inhibition of polypeptide synthesis, and homotypic and heterotypic interference in cell culture. In mice, different DI virus preparations gave a similar level of protection but differed in their post-infection immune status, suggesting that protection of mice takes place by more than one mechanism. This suggestion was supported by examination of infectivity levels in mice following i.n. inoculation of DI virus. Mice treated with DI virus p4 had infectivity levels identical to non-treated mice upto day 2 p.i. but declined thereafter. In comparison, infectivities in DI virus p13a-treated mice were reduced throughout infection. The lack of neutralizing antibody in most protected mice suggests that humoral immune responses play at best a minor role in protection. However, short lived IgM levels have not been measured in protected mice so humoral immune responses cannot be completely excluded. As there is no IgM memory this could account for the failure to detect an adaptive immune response in mice protected by

Table 50 Evidence of biological heterogeneity of DI SFV preparations

1. The ratio of interference titres measured by RSIA:YRA varies from preparation to preparation.

2. Inhibition of polypeptide synthesis in co-infected cells may affect:
   a) virus structural polypeptides and/or
   b) virus non-structural polypeptides and/or
   c) host polypeptides.

3. Homotypic and heterotypic interference vary between preparations.

4. Protection of mice from 10 LD_{50} after i.n. inoculation with different DI virus preparations gives:
   a) protection with strong adaptive immune response or
   b) protection with weak adaptive immune response or
   c) no protection.

5. Protection of mice from 10 LD_{50} after i.p. inoculation with different DI virus preparations gives variable
   a) protection,
   b) delay in death.

6. No correlation between protection by DI virus preparations obtained by the i.n. route and that obtained by i.p. inoculation.

7. No correlation between *in vitro* interference properties and protection of mice by i.n. or i.p. routes.

DI virus p4.

DI virus was also found to modulate the lethal encephalitis after co-inoculation of DI and S virus by the i.p. route. Again, modulation took place by two mechanisms which were exemplified by DI viruses p4 and p13a.

It is concluded from this evidence that DI SFV preparations are biologically heterogeneous and have different interfering properties but the origin of these variations have not yet been fully explored. Sequence analyses by others have shown that SFV DI particle RNA is physically heterogeneous. A p11 DI SFV preparation contained more than one DI RNA species, and these RNAs differed in the extent of virus genome sequences deleted, sequence rearrangements and repetitions of an approximately 300 to 500 nucleotide sequence (Pettersson, 1981; Kääriäinen et al., 1981; Söderlund et al., 1981; Lehtovaara et al., 1981, 1982). Kääriäinen et al. (1981) also observed that the population of DI RNAs varied on passage and this may explain the biological heterogeneity of DI viruses described in this thesis. It is possible that a preparation of DI SFV consists of a population of particles of defined but differing RNA sequences which vary in proportion during passage and this would result in the variation in interfering properties described in this thesis. A further source of variation may well arise from changes in the sequence of individual DI RNAs during replication. Additional opportunity for variation would occur if there was interference between DI viruses in a population as described with DI VSV (Rao and Huang, 1982). The lack of correlation between *in vitro* and *in vivo* interference may be explained by only a minority of DI RNA species

in the population being able to interfere *in vivo*. This suggestion is supported by a preliminary T_1 oligonucleotide mapping study. DI SFV preparation p5 was taken and digested with T_1 ribonuclease and the oligonucleotides separated by PAGE (Figure 21a). The "fingerprint" of DI virus p5 differs little from that of DI virus p8 described by Kennedy (1976) (Figure 21b). Although T_1 mapping only represents 10% of the virus genome it would appear, at a gross level, that the 2 DI viruses are similar. However, DI virus p8 protects mice after i.n. inoculation, while DI virus p5 does not. Thus the above suggests that only some (a minority?) of the DI RNA species in the population will interfere *in vivo*. Thus it can be envisaged that only DI SFV preparations which contain certain DI RNA species in the population will be able to interfere *in vivo*. To analyse biological heterogeneity further it will be necessary to examine structure-function relationships between different DI SFV preparations and the population of DI RNA species within the preparations. This will require T_1 oligonucleotide mapping of DI SFV preparations and quantitation of the molar ratios of oligonucleotides to determine if a preponderance of certain oligonucleotides correlates with particular biological properties.

Figure 21a T_1 oligonucleotide map of DI SFV p5

The lower cross is the bromophenol blue dye marker and the upper cross is the xylene cyanol dye marker.


(a)


(a)

Figure 21b T_1 oligonucleotide map of DI SFV p8 (Kennedy, 1976).

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Appendix

Statistical formulae

i) Standard error of the mean and confidence limits

The mean, $\bar{x}$, is the point about which the distribution is symmetrical and is described by:

$$\bar{x} = \frac{\Sigma x}{n}$$

where x are the values obtained on n number of measurements.

The standard deviation, σ, is a measure of the variability of the measurements about this mean and is described by:

$$\sigma = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

The "standard error" (i.e. the standard deviation of the mean) is described by:

$$S = \frac{\sigma}{\sqrt{n}}$$

Where S = standard error, σ = standard deviation and n = number of measurements.

Therefore the reliability of a sample mean in indicating the true mean of the whole population can be described. It is useful to attach confidence limits at a certain level of probability. Thus, for any normal distribution 95% of measurements will be 1.96 standard deviations either side of the mean (i.e. the mean has a probability of 95% of being

between these limits) or:

$$\bar{x} \pm 1.96 \frac{s}{\sqrt{n}}$$

ii) Statistical analysis of UV inactivation kinetics

Since the process of inactivation is random, it follows the Poissonian distribution. Therefore, the possibility of any single template remaining functional (i.e. unhit) is given by:

$$P_r = \left(\frac{x^r}{r!}\right) \cdot e^{-x}$$

Where P_r = probability of the template having received r hits, where the average is x hits per template.

If the average = 1 hit per template

$$x = 1$$

Therefore, P_r that any single template remains active (i.e. unhit) is r = o.

Therefore $$Pr = e^{-1} = \frac{1}{e} = 0.3679$$

Thus at the time at which there is an average of 1 hit per template 36.8% of the templates are still unhit and involved in RNA synthesis. This time is known as $t_{37\%}$ (Ball, 1977).

II